REVIEW FOR PHYSIOLOGICAL REVIEWS

TRPV4: MOLECULAR CONDUCTOR OF A DIVERSE ORCHESTRA

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Abstract

Transient receptor potential vanilloid type 4 (TRPV4) is a calcium permeable non-selective cation channel, originally identified in 2,000 by research teams led by Schultz **(376)** and Liedtke **(229)**, respectively. TRPV4 is now recognised as being a polymodal ionotropic receptor which is activated by a disparate array of stimuli, ranging from hypotonicity to heat and acidic pH. Importantly, this receptor is constitutively expressed and capable of spontaneous activity in the absence of agonist stimulation which suggests that it serves important physiological functions – as does its widespread dissemination throughout the body and its capacity to interact with other proteins. Not surprisingly, therefore, it has emerged more recently that TRPV4 fulfills a great number of important physiological roles and that various disease states are attributable to the absence, or abnormal functioning, of this ion channel. Here, we review the known characteristics of this ion channel's structure, localisation and function, including its activators, and examine its functional importance in health and disease.

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1. Introduction

Transient receptor potential vanilloid type 4 (TRPV4) ion channels are Ca^{2+} permeable non-selective cation channels which exhibit a remarkable range of expression throughout the human body. TRPV4 belongs to the transient receptor potential vanilloid (TRPV) sub-family of transient receptor potential (TRP) cation channels. Thus far, twenty-eight TRP channels have been identified in mammals and these have been classified on the basis of sequence homology into six sub-families. In addition to the TRPV sub-familly, these sub-families comprise: the TRPC ("Canonical"); the TRPM ("Melastatin"); the TRPP ("Polycystin"); the TRPML ("Mucolipin"); and the TRPA ("Ankyrin") **(157, 194, 421).** The TRPV4 cation channel was first described in 2000 **(229, 380)** and was surprising in that osmo-sensing, volume-regulatory, ion channels had been previously thought to be limited to the volume-regulated anion channel (VRAC) **(292)**. TRPV4 was initially given different names derived from the key features observed by the denominating investigators. Thus, the channel was respectively denominated as the osmosensitive transient receptor potential channel 4 (OTRPC4) **(229, 380)**, the vanilloid receptor-related osmotically activated channel (VR-OAC) **(229)**, the vanilloid receptor like channel 2 (VRL-2) **(88)**, and the transient receptor potential channel 12 (TRP12) **(292, 442)**. However, by 2002, it had been appreciated that the channels thus described were, in fact, the same TRPV4 channel which is activated by a diverse range of stimuli so that it has been well-described as exhibiting "promiscuous gating behaviour" **(289, 296, 297, 320, 421, 432)**.

It is now known that TRPV4 plays a fundamental, and quite general, role in normal physiology and that its malfunction results in various diseases **(109)**. However, as the early nomenclature implies (ORTPC4 and VR-OAC), TRPV4 was initially identified as being of more limited importance as a mediator of responses to changing osmotic conditions in mammals **(228)**. Liedtke and colleagues (2003) showed that mice which do not have the TRPV4 receptor exhibit reduced responses to both hyper-osmolar and hypo-osmolar stimuli. These animals consume less water and become more hyperosmolar than normal animals. Antidiuretic hormone levels in the blood of TRPV4-null mice are also reduced when compared to normal animals which have been subjected to a hyperosmotic challenge. Moreover, TRPV4-null animals experience systemic hypotonicity on continuous subcutaneous infusion of the antidiuretic hormone analogue, deamino-8-D-arginine

vasopressin (dDAVP), although their renal water reabsorption capacity remains normal **(230)**. At the single cell level, TRPV4, in certain at least of those cells where it is expressed, contributes to regulatory volume decrease (RVD) **(32, 33, 309)**. However, the nature and extent of the involvement of TRPV4 in osmoregulation remains very much in the course of elaboration as do the respective roles of the various other components of that complex system **(365).**

It is now recognised that TRPV4 function extends considerably beyond its role in osmoregulation and that this ion channel is involved in mediating a diverse array of physiological and pathological conditions. TRPV4 ion channels perform an essential role in regulating the functioning of the cell by virtue of their being Ca^{2+} influx channels in the cells in which they are expressed **(382, 420)**. It is this capacity of TRPV4 to regulate intracellular calcium concentration ($[Ca^{2+}]_i$) in the manner, and to the extent, to which it does, and in diverse contexts, which enables TRPV4 to regulate a diverse orchestra of intracellular functional proteins which are essential for supporting the physiological processes required for maintaining homeostasis. A revealing indicator of the physiological importance of TRPV4 is found in its role in the vascular endothelium, where it contributes to intracellular Ca^{2+} homeostasis and regulation of cell volume while responding to mechanical stresses on the vasculature **(167)**. Ca^{2+} influx mediated by activation of endothelial TRPV4 maintains shear stress-induced arterial vasodilation **(158)** and is essential for the regulation of arteriolar tone, as well as membrane permeability and the production of antithrombotic factors **(22, 374, 375, 386)**. Strikingly, excessive activation of this ion channel occasions circulatory collapse resulting from endothelial cell activation and damage as well as from leakage of the pulmonary microvascular permeability barrier **(441)**. Not surprisingly therefore, failure of normal TRPV4 expression and function is responsible for various disease states but, remarkably, this ion channel is also implicated in the aetiology of several hereditary channelopathy-induced diseases **(295)**. Thus, mutations in the TRPV4 gene have been shown to result in the genetic disorders of brachyolmia (a syndrome principally characterised by short-trunk dwarfism), as well as various autosomal-dominant skeletal dysplasias and neuromuscular diseases, including Charcot-Marie-Tooth disease type 2C (CMT 2C), spinal muscular atrophy (SMA) and hereditary motor and sensory neuropathy type 2 (HMSN 2C) **(81, 295, 414)**. Of further interest, TRPV4 has been shown to play an important role in mediating certain pain sensations, with TRPV4 being an essential contributor to inflammation-induced "mechanical hyperalgesia" **(8, 12, 70, 72, 89, 476, 477)**, and also

being involved in mediating pain in several types of neuropathy **(9, 10, 73, 94, 428)**. Again, autophagy is an essential degradation system within the cell that causes cytoplasmic components to be delivered to the lysosome, entailing sequential steps comprising sequestration, subsequent transfer to lysosomes, then degradation, and re-use of the products of such degradation **(271)**. TRPV4 has been found to be involved in the regulation of apoptosis and autophagy in hepatic stellate (HSC-T6) cells. When transfected with small interfering RNA (si-TRPV4) to prevent the expression of TRPV4 (si-TRPV4), these cells exhibit an increase in apoptosis and inhibition of autophagy. On the other hand, when these cells are treated with the TRPV4 agonist, 4α-phorbol 12,13-didecanoate (4α-PDD), the result is reduction of apoptosis and an increase in autophagy **(466)**. It is already abundantly clear that the multiplicity of pathophysiological conditions in which TRPV4 has been implicated suggests that this ion channel may prove to be an immensely important target for therapeutic pharmacological intervention **(293)**. However, such targeting of TRPV4 is complicated by the risk of compromising the important physiological functions which this ion channel subserves.

Once regarded as a Cinderella amongst ion channels, functioning only in the maintenance of systemic osmotic equilibrium, TRPV4 is now credited as being a major player in human physiology, crucially functioning not only in the maintenance of vascular tone and endothelial cell function, but also in the cardiac, respiratory, urinary, skeletal and digestive systems, as well as in the central and peripheral nervous systems, while also being responsible for various channelopathy-induced hereditary diseases and pain of several different contexts of origin. These diverse aspects of the applied physiology of TRPV4 are afforded consideration in their turn in the later part of this Review; but, first, we address fundamental issues relating to this ion channel: the *Trpv4* gene, the protein's structure; the pattern of its expression; what is known about how it functions in relation to its structure and in relation to other associated proteins; how it is activated; its antagonists; and its role as an interactive molecular integrator of diverse stimuli.

2. *Trpv4* gene

Directions for the manufacture of the human TRPV4 ion channel are provided by the *Trpv4* gene which belongs to both the ANKRD (ankyrin repeat domain containing) and the

TRP (transient receptor potential cation channel) families of genes. The TRPV4 gene is found on the long (q) arm of chromosome 12 at position 24.1, being located from base pair 109,783,086 to base pair 109,833,406 on chromosome 12 (**(88)**; http://ghr.nlm.nih.gov/gene/TRPV4; **Figure 1**). There is evidence that microRNA-203 (miRNA-203) exercises control over this gene in rat **(172)**. Progesterone reduces expression of TRPV4 in human airways, mammary gland epithelial cells, and in vascular smooth muscle cells **(190)**. When rats suffering from visceral hypersensitivity, resulting from neonatal maternal separation, are given a combination of 8 probiotic bacteria strains (comprised in the medical food VSL#3), their visceral pain is reduced while a variety of genes involved in pain and inflammation are found to be down-regulated, including TRPV4 **(95)**. Other factors identified as increasing TRPV4 expression are interleukins 1*B* and 17 (in DRG neurons), NGF (in urothelium) and hypoxia/ischaemia (in astrocytes and pulmonary arterial smooth muscle cells) **(48, 132, 138, 343, 447)**.

3. TRPV4 Protein Structure and Function

(i) Relevance of structural data derived from TRPV1

The characterisation of the molecular structure of TRPV4 is very much a work in progress. TRPV1 is by far the best-characterised member of the vanilloid sub-family TRPV1-TRPV6 and its features have thus far been elaborated in much greater detail than those of TRPV4 (**Figure 1(E)**). A major advance in the elucidation of TRPV1 structure was recently made by Liao and colleagues (2013). These researchers managed to overcome the obstacles inherent in investigating the structure of a small membrane-bound molecule which is likely to be the subject of a high level of ongoing conformational change as suggested by the number and diversity of its potential activators **(54, 163, 191, 226, 227)**. The findings which have been made in relation to TRPV1 protein structure are relevant in the context of TRPV4 because there is high confidence that many of the features of TRPV1 are shared by TRPV4. Indeed, it is generally accepted that many of the *lacunae* in our knowledge of the molecular structure of TRPV4 can be reliably filled by structural data from studies of TRPV1 when considered in conjunction with studies of the evolutionary profiles of TRPV1-TRPV4 **(96, 291)**. It has been suggested that the several features of the TRPV1-TRPV4 ion channels, namely, the ankyrin repeat domains (ARDs), membrane proximal domains (MPD), trans-membrane domains (TMD) and transient receptor potential (TRP) domains (including TRP

box) all exhibit the same basic functional scaffold arrangement from the ARD to the TRP domain across TRPV1-TRPV4. On the other hand, it has also been suggested that the distal N-terminus and the distal C-terminus, as well as certain extracellular loops are highly divergent and specific for individual TRPV1-TRPV4 channel types **(96)**.

(ii) Structure of the TRPV4 protein

The *TRPV1 protein* in rat is made of 838 amino acids, while that in the human comprises 839 amino acids. The relative molecular mass of the TRPV1 molecule is predicted to be 95,000 Da **(57)**. The TRPV1 protein itself is a multi-molecular structure in which its component molecules are arranged to constitute an ion channel. TRPV1 is an oligomer which comprises four monomers. The protein structure is therefore tetrameric, demonstrating a four-fold symmetry around a central aqueous pore **(273)**. Individual molecules in the TRPV1 tetrameric protein are usually referred to as TRPV1 "channel sub-units." When expressed, these individual molecules have a specific protein component which enables them to combine to constitute a functional ion channel. Each monomer has six transmembrane alpha-helices (S1-S6) which extend across the lipid bilayer, together with a re-entering loop which contains the pore helix found between S5-S6. N- and C-termini are localised in the cytoplasm. A helical S4-S5 linker runs parallel to the membrane and connects the S1-S4 and S5-P-S6 segments within each TRPV1 subunit **(226)**. These features of the TRPV1 protein are afforded further consideration in the succeeding paragraphs.

The *TRPV4 protein* is known to be composed of 871 amino acid residues (**(380)**; **Figure 2**) and possesses the same tetrameric structure **(358)**. TRPV4 is normally assembled as a homotetramer **(378)**, although heterotetramers have also been reported, e.g., with TRPC1 **(244)**, TRPP2 **(378)**, and even heteromeric TRPV4-TRPC1-TRPP2 complexes **(98)**. Each TRPV4 monomer has six transmembrane alpha-helices (S1-S6) stretching across the lipid bilayer, together with a re-entering loop which contains the pore helix found between S5-S6. The transmembrane region is similar to that of TRPV1. N- and C-termini are localised in the cytoplasm **(358)**.

(a) The N-terminus

The *N-terminus of TRPV1* possesses six ankyrin repeat domains (ARDs) (aa 101–364). These are 33-residue sequence motifs, which are frequently involved in protein-protein

interactions and are necessary for the channel to function (**(164, 189, 226, 232, 250, 279)**; **Figure 2(E)**). Liao and colleagues describe how residues in ARDs 3 and 4 from one subunit interact with a three-stranded anti-parallel *B*-sheet from an ARD-S1 linker region (K368-D383) and the C-terminus of an adjacent subunit to press the cytoplasmic component of TRPV1 together. In addition, this *B*-sheet structure anchors the cytoplasmic N- and C-terminal domains together within the same subunit. Residues G375 and P376 are found in all TRPV subtypes and produce a sharp turn which connects the N-terminal *B*-strands **(226)**. The N-terminus of the TRPV1 molecule is also involved in interaction with some auxiliary proteins including snapin and synaptotagmin IX **(277)**. Molecular modelling of human TRPV1 suggests that when the channel has been desensitised, the N-terminal becomes more proximate to the C-terminus **(117)**. The ARDs of the TRPV1 molecule also comprise a binding-site for ATP and a calcium-binding protein, calmodulin. ATP and calmodulin have opposing roles in the desensitisation process in TRPV1 **(232)**.

This ARDs' binding site for ATP and calmodulin (CaM) is conserved in TRPV3 and TRPV4, but not in the TRPV2 molecule **(319)**. Binding of phosphoinositides to a TRPV4 ARD results in a reduction of channel function. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) binds more potently to the TRPV4 ARD than do other phosphoinositides. The TRPV4 ARD has been characterised as a lipid-binding domain which interacts with PI(4,5)P2 to regulate the activity of TRPV4 **(395)**. Deletions from the N-terminus region are involved in a number of splice variants. Arniges and colleagues (2006) describe five splice variants of the TRPV4 channel: TRPV4-A andTRPV4-B (delta384-444) variants, TRPV4-C (delta237-284), TRPV4-D (delta27-61), and TRPV4-E (delta237-284 and delta384-444) variants. These alternative splice variants all entail deletions in the cytoplasmic N-terminus region, affecting (except for TRPV4-D) the ankyrin domains. They assign these variants to two groups: Group I which comprises TRPV4-A and TRPV4-D; and Group II which comprises TRPV4-B, TRPV4-C, and TRPV4-E. The variants in Group I (unlike those in Group II) are correctly processed, homo- and heteromultimerized in the endoplasmic reticulum, and targeted to the plasma membrane where they respond to typical TRPV4 stimuli. Group II variants, on the other hand, do not have parts of the ankyrin domains normally posessed by TRPV4, cannot oligomerize, and remain within the cell in the endoplasmic reticulum (**(19)**; **Figure 3**). The cytoskeleton protein PACSIN 3 interacts with a proline-rich region just upstream of the first ARD **(79)**. ARDs are important in channel synthesis. Studies on TRPV4 suggest that ion channel synthesis involves core glycosylation

and oligomerization in the endoplasmic reticulum with subsequent transfer to the Golgi apparatus for maturation. ARDs are necessary for such oligomerization **(19).** In addition to ARDs, other parts of the N-terminus (residues 64–77) in TRPV channels may also be involved in channel assembly **(63)**. **Correct channel assembly of TRPV4 requires an interaction between the pre-S1, TRP, and the ARD-S1 linker domains. When the pre-S1 K462 of TRPV4 is neutralised, the result is that TRPV4 protein is retained in the ER, and there is impaired glycosylation and trafficking, while TRPV4 activators fail to elicit an appropriate response from the channel (131).**

The N-terminus of TRPV4 has a proline-rich region which is important in mediating the effects of PACSIN 3 (Figure 2(G) & 5) which binds to TRPV4 to regulate the subcellular localisation of TRPV4 as well as to potently inhibit the basal level of activity of the ion channel and its activation by cell swelling or heat, but not its activation by 4α-PDD. Interaction of PACSIN 3 with TRPV4 requires both this proline-rich domain located before the ankyrin repeats of the TRPV4 N-terminus as well as the PACSIN 3 C-terminus Src homology 3 domain. When mutations are made at certain proline residues found in the N-terminus of TRPV4, the ion channel is saved from such inhibition of its activity by PACSIN 3 **(79, 93)**.

(b) The C-terminus

The *C-terminus of TRPV1* is required for normal channel function and damage to this region can occasion major functional deficits **(41, 419)**. A segment in the C-terminus (aa 684-721) contains the *TRP domain*, which is adjacent to the channel gate and is essential for the tetramerisation of the channel subunits into functional channels **(134)**. This *TRP domain* is involved in interactions between the constituent subunits of oligomeric TRPV1 and contributes to the linking of stimulus sensing to channel gating **(135)**. Liao and colleagues describe the TRP domain as comprising a 23-25 amino acid segment found immediately after S6 which has an alpha-helical structure that extends parallel with the membrane's inner leaflet as a result of an acute bend after S6. The alpha-helix embraces the initial two-thirds of the *TRP domain* before it is replaced by a random coil. The *TRP domain* also contains charged side chains on the cytoplasmic side of the helix which interact with a pre-S1 helix by means of hydrogen bonding and salt bridging **(226)**. The *TRP box* describes a highly conserved, 6-mer segment in the C terminus, close to the channel gate, which is found in TRP channels within the TRP domain. In TRPV1, mutation of this segment interferes with normal

gating of the channel and it can alter the response of TRPV1 to voltage, capsaicin, and heat **(410)**. It has been proposed that interactions either between, and/or within, subunits at the TRP box are essential for the proper linking of stimulus sensing to gate opening **(148)**. A segment comprising R701 and T704, located beside the TRP box, is considered to function in decelerated gating associated with desensitisation of TRPV1 **(302)**. A CaM-binding motif is also attributed to five residues in the C terminus which putatively constitute an alpha helix to connect into the central cavity of calmodulin **(149)**.

The *C terminus region of TRPV4* is predicted to be structurally similar to that of TRPV1 and to be located to the surface, rather than to the inside, of the cytoplasmic region of TRPV4 **(358)** . Channel protein folding, maturation and trafficking are dependent on the C-terminus component of TRPV4; and the segment which includes residues 838-857 is critical in enabling these processes to occur. These residues 838-857 adopt a precise conformation, as demonstrated by structural modelling, with Gly849 and Pro851 becoming localised at essential positions **(222)**. The TRPV4 C-terminus interacts with the microtubule-associated protein 7 (MAP7) **(387)**. A PDZ binding-like sequence is found in the final four amino acid residues of the TRPV4 C terminus **(130)**. A single mutation at E797 in the C-terminus of TRPV4 results in the channel becoming constitutively open **(433)**.

(c) The pore region

The *pore domain* of TRPV1 is comprised of two transmembrane domains – S5 and S6 – and the loop between them. Salazar and colleagues (2009) showed that S6 opens the pore of TRPV1 as a result of binding by capsaicin or temperature increase. The S6 segments constituting the pore are helical in structure, while two constrictions are found in the pore. One of these restricts entry by large molecules, while the other impedes the ingress of smaller ions and constitutes an activation gate of TRPV1 **(336)**. The S6 segment of TRPV1 comprises a watery hollow within an amphipathic alpha helix, with the majority of the hydrophobic residues oriented into the cavity **(179)**. The study of the TRPV1 channel in its closed state by Liao and colleagues (2013) identifies the outer pore of TRPV1 as having a broad funnel-like structure while a short selectivity filter (^{643}GMGD^{646}), with backbone carbonyls or side chains facing into the central entry-way, is found deeper into the central canal. Diagonally opposed carbonyl oxygens at G643 form a restriction point which is consistent with the selectivity filter being in a non-conducting state. The most constricted point in the lower gate is constituted by a constriction site deeper into the canal where a

hydrophobic seal of 5.3A is formed by the combination of I679 in the S6 helices from each subunit. Y671 and L681 are unlikely to amount to important contributors to the lower gate as side chains of Y671 are found ~10A above the most constricted point made by I679, while the side chain of L681 is oriented away from the conducting channel of the pore. TRPV subtypes are highly similar in sequence within the S6 helix. Moreover, each TRPV subtype has an isoleucine located at a position which is the equivalent of I679 in TRPV1, which strongly suggests that the lower gate in each TRPV subtype exhibits a similar constitution **(226)**. Gating of TRPV1 involves substantial re-arrangement of the structure of the outer pore as well as the pore helix and the selectivity filter, in addition to a significant reduction in the extent of the hydrophobic constriction found at the lower gate. However, the S1-S4 domain of TRPV1 does not appear to be active during gating of the channel **(54)**. Simulations using molecular dynamics indicate that the TRPV1 pore has three main binding sites for cations. One of these binding sites is associated with the pore's intracellular cavity, while the remaining two are found at the interacellular and extracellular entries to the selectivity filter. The intracellular cavity is described as representing a broad attractive area which permits cations to diffuse freely. Cations are subjected to electrostatic attraction at the outer entrance to the selectivity filter by reason of the presence of an arrangement of acidic residues in two rings constituted by E648 and D646. TRPV1 appears to have a very flexible selectivity filter whereby its structure differs with the peameation of Na^+ and K^+, respectively. Additional conformational changes of the selectivity filter are believed to be necessary to permit the permeation of Ca^{2+}**(83)**.

A consensus N-linked glycosylation motif lies within the loop which forms the pore between the fifth and sixth transmembrane segments of TRPV4 **(449)**. Asp672, Asp682 and Met680 are components of the channel pore of TRPV4; and neutralizing either of these aspartate residues to alanine results in a moderate reduction in the permeability of TRPV4 to divalent cations and a similar reduction in the extent of outward rectification – a double effect which is increased by neutralisation of both of these residues concurrently. Met680 is a putative component of the pore's selectivity filter and its mutation strongly reduces whole cell current amplitude as well as the permeability of the channel to Ca^{2+} **(420)**. Mutation of E797 in the C-terminus of TRPV4 causes the pore to become constitutively open **(433)**.

(iii) Activation sites

4α-phorbol esters have their binding site in a loop in the 3rd and 4th transmembrane domains of TRPV4. Activation is dependent on the ester decoration of ring C and the A,B ring junction. The lipophilic ester groups on ring C function principally to effect the positioning of the diterpenoid core into the ligand binding area **(201)**. Sensitivity of TRPV4 to 4α-PDD and heat is diminished by the substitution of leucine for F617, Y621 or F624 in TM5 but, remarkably, the resulting F617L and Y621L mutants exhibit enhanced activation due to cell swelling. A Y702L mutation in TM6 diminishes sensitivity to 4α-PDD, heat and cell swelling. Thus, there are distinct pathways which mediate the response of TRPV4 to 4α-PDD and changes in osmolarity, respectively **(200)**. Two hydrophobic residues in the middle area of the fourth transmembrane segment (TM4; Leu584 and Trp586) govern the sensitivity of the channel to 4α–PDD, bisandrographolide A, and heat, but have no effect on the responses to cell swelling, arachidonic acid and epoxyeicosatrienoic acid (5,6 EET). However, two residues in the C-terminal area of TM4 (Tyr591 and Arg594) are implicated in the activation of TRPV4 by all of these stimuli **(425)**. Nitric oxide (NO) causes the S-nitrosylation of TRPV4 on the Cys853 residue to reduce the activation of TRPV4 (Lee et al., 2011). Tyrosines 110 and 805 have been identified as phosphorylation sites of Src kinase in TRPV4 with the Tyr110 residue proposed to be important in the stimulus-specific modulation of TRPV4 channel function (Wegierski et al., 2009) **(437)**. The Ser824 residue of TRPV4 is phosphorylated by glucocorticoid-induced protein kinase 1 (SGK1) and phosphorylation of this residue is required for the interaction of TRPV4 with F-actin **(361)**. A voltage-dependent gating mechanism has been proposed to exist in series with the principal intracellular gate **(238).** The N-tail has a binding site for PI(4,5)P2, while channel activation by physiological stimuli is enabled by the PI(4,5)P2-dependent rearrangement of the cytosolic tails of TRPV4 **(133)**. The C-terminus of TRPV4 has an intracellular CaM binding domain and this constitutes the structural component for the effect of Ca^{2+} in the activation of the channel **(381)**. All three members of the PACSIN protein family can bind the amino terminus of TRPV4 in mouse **(79).**

(iv) Interactions of TRPV4 with other proteins

Thus far, 38 interacting proteins have been identified for TRPV4 (http://trpchannel.org/summaries/TRPV4; **Figure 5**). The function of many of these

interacting proteins has yet to be elucidated, but it is possible to categorise those whose functions are (in part at least) known into several broad categories (which are not mutually exclusive) for the purpose of facilitating this discussion. On this practical basis, we consider these interacting proteins under four heads, namely: (a) proteins which interact with TRPV4 to modify the localisation of TRPV4; (b) cytoskeletal proteins which interact with TRPV4; (c) proteins which interact with TRPV4 to modulate signalling; and (d) ion channel proteins which interact with TRPV4, including those which interact to form heteromeric TRPV4 channels.

(a) Proteins interacting with TRPV4 to modify TRPV4 localisation

Several proteins modify the localisation of TRPV4, including PACSIN 3, OS-9 and ubiquitin ligase AIP4. PACSIN 3 is an auxiliary protein of TRPV4 and regulates the subcellular localisation of TRPV4 by affecting its endocytosis, as well as regulating its activity in a stimulus-specific manner. The localisation of TRPV4 as between the plasma membrane and cytosol is influenced by the co-expression of PACSIN 3, although not by either of the other PACSINs. When PACSIN 3 is also expressed with TRPV4, the extent to which TRPV4 is expressed in the plasma membrane increases at the expense of its expression in the cytosol. The localisation of TRPV4 at the subcellular level reflects the influence of PACSIN 3 on the endocytosis of TRPV4. The interaction of TRPV4 and PACSIN 3 requires a carboxyl-terminal Src homology 3 (SH3) domain of PACSIN 3 and a specific proline-rich domain upstream of the ankyrin repeats of TRPV4 (**(79)**; **Figure 4**). PACSIN 3 strongly inhibits the basal activity of TRPV4, as well as its activation by cell swelling and heat; but PACSIN 3 has no effect on the gating of the channel induced by 4α-PDD. This inhibitory effect on TRPV4 is abolished by a single proline mutation in the SH3 domain of PACSIN 3 which implies that PACSIN 3 binds to TRPV4 to regulate its activity **(93)**. The N-terminus tail of TRPV4 located in the cytosol interacts with a protein associated with the endoplasmic reticulum (ER) known as OS-9. OS-9 functions to reduce the release of TRPV4 from the ER membrane by binding preferentially TRPV4 monomers and other non-developed variants of this ion channel which are localised there. This enables OS-9 to reduce the level of TRPV4 found at the plasma membrane **(431)**. Ubiquitination is an important factor in the regulation of TRPV4 expression at the plasma membrane. Increased ubiquitination of TRPV4 is mediated by the HECT (homologous to E6-AP carboxyl terminus)-family ubiquitin ligase

AIP4 but without inducing the degradation of TRPV4. This ubiquitin ligase AIP4 instead facilitates the endocytosis of TRPV4, thereby reducing the extent of its expression at the plasma membrane. In the result, while TRPV4 levels are increased, there is nevertheless a reduction in the basal activity of the ion channel **(436)**. *B*-arrestin 1 interacts with AIP4 to produce the ubiquitination of TRPV4 **(390).**

The stromal interaction molecule 1 (STIM1) is a type 1 transmembrane protein that enables Ca^{2+} influx to occur after intracellular Ca^{2+} stores have become depleted. It functions by gating store-operated Ca^{2+} influx channels. STIM1 functions as an auxillary protein of TRPV4 channels expressed in epithelium where it complexes with the C-terminus tail of TRPV4. Phosphorylation of serine 824 of TRPV4 controls the formation of the TRPV4/STIM1 complex and its localisation in the cell membrane. It has been proposed that the TRPV4/STIM1 complex is essential for guiding TRPV4 from the endoplasmic reticulum to the cell membrane and for its proper functioning **(360)**.

(b) Cytoskeletal proteins interacting with TRPV4

TRPV4 and actin are strongly co-expressed in some membrane structures. These are highly dynamic structures and include microvilli, filopodia and lamellipodia edges as found in HaCaT keratinocytes and CHO cells. Moreover, TRPV4 and F-actin interact and function in sensing hypotonicity and the development of RVD **(32)**. Goswami and colleagues (2010) made remarkable observations concerning the physical interaction of TRPV4 with tubulin, actin and neurofilament proteins, as well as with PKCε and CamKII in DRG neurons and in DRG-neuron-derived F-11 cells. The C-terminus of TRPV4 interacts directly with tubulin and actin which compete for binding. When TRPV4 are activated, alteration in the morphology of neuronal and non-neuronal cell types is induced. At the same time, microtubule activity exercises control over the functioning of TRPV4 **(142)**. TRPV4 is localised with caveolin 1 and filipin in a lipid raft. Both Loop 4 and the TM5-Loop 4-TM5 area of TRPV4 are capable of physically interacting with cholesterol, as well as mevalonate, its precursor, and derivatives including stigmasterol and aldosterone **(207)**.

*B*5-tubulin is one of at least seven *B*-tubulin isotypes produced in vertebrates that assemble together to form cellular microtubules. Over-expression of *B*5-tubulin can severely damage the microtubule network. Annexin A2 is a protein which acts as an autocrine factor to increase osteoclast formation and bone resorption. TRPV4 is found co-localised with *B*5-

tubulin and annexin A2 in transfected HEK293 cells, but only TRPV4 and *B*5-tubulin interact to form a complex **(174)**. An association of annexin A2 with TRPV4 has also been found in DRG neurons together with evidence that annexin A2 is involved there in the regulation of TRPV4-mediated Ca^{2+} influx and substance P release in those neurons **(298)**.

(c) Proteins interacting with TRPV4 to modulate signalling

The arrestins comprise a small group of proteins which function in the regulation of signal transduction. Shukla and colleagues (2010) have demonstrated that ubiquitination and functional down-regulation of TRPV4 are mediated by β -arrestin 1. When *B*-arrestin 1 is stimulated by angiotensin, it becomes attached to a constitutive complex in the cell membrane formed by the angiotensin receptor ($AT_{1a}R$) and TRPV4. *B*-arrestin 1 interacts with an E3 ubiquitin ligase (AIP4) to result in the ubiquitination of TRPV4 and its internalisation and functional down-regulation **(362)**. The progesterone receptor also functions in the regulation of TRPV4 expression, with TRPV4 expression in human airways, mammary gland epithelial cells, and vascular smooth muscle cells, being reduced on exposure to progesterone **(190)**. TRPV4 channels and connexins colocalise with caveolin-1 (the structural protein of caveolae) in the caveolar compartment of the plasma membrane of mouse endothelial cells and TRPV4 activity is reduced in the absence of caveolin 1 **(337)**. **The satellite glial cells (SGCs), which closely ensheath sensory neurons, influence their excitability in conditions involving inflammation and pain. These SGCs express functional TRPV4 as evidenced by the presence of these ion channels in a subpopulation of neurons in mouse DRG. The TRPV4 so expressed appear to be functionally regulated by the $P2Y_1$ receptor which has been proposed to couple to, and activate, TRPV4 (329).**

TRPV4 may perform functions in one sex that it does not in the other. Thus, studies of endothelial function in isolated porcine coronary arteries show that, while no difference in the level of expression of TRPV4 is found using Western blot as between male and female, the TRPV4 antagonist, RN-1734, diminishes the strength of the NO component of the vasorelaxation induced by bradykinin in females but not in males, while inhibiting in both sexes the maximum relaxation induced by the endothelium-dependent hyperpolarisation-type component **(443)**.

It has already been observed that several enzymes affect the activity of TRPV4. Thus, enhancement of the activation of TRPV4 by phosphorylation of specific sites is dependent upon AKAP79 assembling PKC or PKA to constitute a signalling complex with TRPV4 **(112)**. TRPV4 can be sensitised by PKC in central sensory and non-sensory nerve terminals, as well as in DRG neuronal cell bodies **(53)**. Annexin A2 is associated with TRPV4 in DRG and there is evidence that it is involved there in regulating TRPV4-mediated Ca^{2+} influx and substance P release **(174, 298)**. TRPV4 and TRPV1 are found together in a population of DRG neurons **(53)**. Both TRPV1 and TRPV4 are also expressed in rat intrapulmonary arteries and their agonist-induced activation results in pulmonary arterial smooth muscle cell migratory responses which are associated with re=organisation of the F-actin cytoskeleton and the tubulin and intermediate filament networks. Proliferation and migration of pulmonary arterial smooth muscle cells leads to increased pulmonary vascular resistance characteristic of pulmonary hypertension **(257)**. TRPV4 interacts with F-actin in sensing hypotonicity and in effecting RVD **(32)**. The association and surface expression of aquaporin 5 (AQP5) and TRPV4 is increased by hypotonicity, although actin depolymerisation reduces these effects and RVD. AQP5 – and not cell swelling *per se* – is essential for the gating of TRPV4 by hypotonicity; and TRPV4 and AQP5 act in concert to control RVD **(234)**. Aquaporin 2 (AQP2) is also involved in the activation of TRPV4 by hypotonicity in renal cells and regulation of the cellular response to osmotic stress, suggesting that both proteins are assembled in a signalling complex that responds to anisosmotic conditions **(125)**.

(d) Ion channel proteins which interact with TRPV4

The closest interaction of other ion channels with TRPV4 is found where those ion channels comprise monomers which are assembled with TRPV4 monomers to form heteromeric TRPV4 channels. The transient receptor potential canonical type 1 (TRPC1) channel and the transient receptor potential canonical type 6 (TRPC6) channel are stretch-activated channels which are frequently found with TRPV4 in DRG neurons where they combine with TRPV4 channels to occasion the sensitisation of primary afferent nociceptors and the occurrence of mechanical hyperalgesia **(7)**. In human HEK293 cells transfected with both TRPV4 and TRPC1 and in native vascular endothelial cells, the loss of stored Ca^{2+} provokes the insertion of TRPV4-C1 heteromeric channels into the plasma membrane. These

TRPV4-C1 heteromeric channels are preferentially localised to the cell membrane when compared to the extent of the translocation of TRPC1 or TRPV4 homomeric channels achieved **(243)**. The polycystin-2 (TRPP2) ion channel uses TRPV4 to form a mechano-sensitive and thermosensitive molecular sensor in the cilium **(205)**. TRPP2 and TRPV4 assemble identically to TRPP2-TRPC1, forming a heterotetramer with a 2:2 stoichiometry and an alternating subunit arrangement **(378)**. TRPV4-TRPC1-TRPP2 channel complexes have been found in endothelial cells of cultured rat mesenteric arteries and in HEK293 cells when co-transfected with TRPV4, TRPC1 and TRPP2. Functionally, the channel complexes thus constituted by these assemblies, when found in cells of the vascular endothelium, are activated by flow to mediate Ca^{2+} influx **(98).**

TRPV4 may also interact indirectly with other Ca^{2+}-sensitive proteins located close to TRPV4 channels within signalling microdomains. Ca^{2+}-sensitive large conductance K^+-channels (BK channels) function in conjunction with TRPV4 in both vascular smooth muscle and bronchial epithelium **(103, 118)**. In the former case, amplification of the Ca^{2+}-signal through ryanodine-receptor mediated Ca^{2+}-store release seems to be important in the signalling process **(80, 103)**. TRPV4 is found co-localised with BK channels in the endothelium of mesenteric resistance arteries and with CGRP in sensory neurons **(128)**. TRPV4 physically interacts with KCa2.3 in the endothelial cells of rat mesenteric arteries where acetylcholine and 4α-PDD principally act through a TRPV4-KCa2.3 pathway to induce smooth muscle hyperpolarization and vascular relaxation **(245)**. TRPV4 channels functionally couple to low conductance KCa channels to mediate osmosensing in parvocellular neurones of the paraventricular nucleus of the hypothalamus which is involved in the regulation of cardiovascular as well as renal function **(115)**. Gating of individual TRPV4 channels within a four-channel cluster in the vascular endothelium of resistance arteries is co-operative, with activation of as few as three channels per cell causing maximal dilation through activation of endothelial cell intermediate conductance, and small conductance, Ca^{2+}-sensitive K^+ channels **(374)**. Mutations of the MLC1 gene are associated with megalencephalic leucoencephalopathy with subcortical cysts. MLC1 is over-expressed in astrocytoma cells where it is mainly localised in the plasma membrane and forms part of the Na^+, K^+-ATPase-associated molecular complex that includes the K^+ channel KIr4.1, syntrophin and aquaporin 4 and functionally interacts with TRPV4 **(212)**. TRPV4 is richly expressed in the apical membrane of epithelial cells of the choroid plexus where it interacts physically and functionally with anoctamin 1 (ANO1) which is a Ca^{2+}-activated Cl^-

channel **(396)**. TRPV4, TRPC1 and KCa1.1 interact physically to constitute a triplex in human internal mammary arteries with TRPC1 constituting the linker by means of which TRPV4 and KCa1.1(α) are enabled to interact. 11,12-epoxyeicosatrienoic acid (11,12-EET) acts on this TRPV4-TRPC1-K_{Ca}1.1 triplex to cause hyperpolarisation of smooth muscle cells and vascular relaxation in these arteries **(248)**.

(v) Molecular structure-function correlates

TRPV4 is a Ca^{2+} permeable non-selective cation channel which exhibits both inward and outward rectification, voltage-dependent block by ruthenium red, a moderate selectivity for divalent over monovalent cations, and an Eisenman IV permeability sequence **(420)**. TRPV4 is more permeable to Ca^{2+} than to Ba^{2+} and Sr^{2+}. Both spontaneous and agonist-induced (caused by hypotonic solutions or phorbol esters) TRPV4 currents are substantially diminished at all potentials without the presence of extracellular Ca^{2+}. During agonist-induced activation of the channel, the rate and extent of channel activation is increased by Ca^{2+} entry. Release of Ca^{2+} from intracellular stores can reinstate TRPV4 activity when extracellular Ca^{2+} is absent. **Site-directed mutagenesis suggests that the C-terminus CaM-binding domain is a prerequisite for Ca^{2+}-induced potentiation of TRPV4 in certain expression systems (381). However, the N-terminus ARDs of TRPV4 are also known to possess a CaM-binding site and this too may function in Ca^{2+}-induced modulation of TRPV4 channel activity in other systems.** A molecular model proposed by Strotmann and colleagues (2010) proposes that, in the resting state, an autoinhibitory complex is formed between an N-terminus intracellular domain of the TRPV4 channel and a C-terminus domain comprising a binding site with a high-affinity for CaM. Binding of CaM consequent on increases in $[Ca^{2+}]_i$ displaces the N-terminus domain which may then interact homologously with the same domain of a second subunit **(382)**.

Voets and colleagues (2002) found that the Ca^{2+} selectivity of the TRPV4 pore is influenced by two aspartate residues, Asp672 and Asp682. When either aspartate is neutralised to alanine, there is a modest decrease both in the relative permeability for divalent cations and in the extent of outward rectification. When both aspartates are neutralised at the same time, the extent of the decrease in Ca^{2+} permeability is much greater as is the reduction in channel rectification. In addition, the permeability order for monovalent cations is changed toward Eisenman sequence II or I. Neutralizing Asp682, but not Asp672, results in

a substantial diminution in the affinity of the channel for ruthenium red. A substantial diminution in the amplitude of whole cell currents and loss of permeability to Ca^{2+} occurs in the case of mutations to Met680, which is found in the middle a supposed selectivity filter; but there is no evidence of an alteration in the properties of the pore (believed to be localised at Lys675) being effected by neutralisation of the only residue which is positively charged in that region **(420)**. One mutation at its C terminus (E797 to E797A or E797K) suffices to render the TRPV4 channel constitutively open **(433)**.

PI(4,5)P2 is found in cell membranes and various signaling proteins are derived from it. PI(4,5)P2 is important for the proper functioning of TRPV4 and the role of this phospholipid in TRPV4 channel regulation has been explored by Garcia-Elias and colleagues (2013) who identified a PI(4,5)P2 binding site within the N-terminus. PI(4,5)P2 binds and rearranges the cytosolic tails of TRPV4 to enable hypotonic and heat stimuli to activate the channel; but 4α-PDD, which binds directly to transmembrane domains, activates TRPV4 independently of PI(4,5)P2 **(133)**. The osmotransducing cytosolic messenger EET activates TRPV4 **(434)**, and this is facililtated by PI(4,5)P2 which also enables activation of TRPV4 by heat in inside-out patches **(133)**. The ankyrin repeat domain found in TRPV1, TRPV3 and TRPV4 possesses a ligand binding site for ATP and calmodulin, as well as for other ligands. ATP and calmodulin function through this multi-ligand binding site to mould the varying sensitivity and adaptation profiles of these several ion channels. ATP and CaM function interactively to determine receptor sensitivity to changes in Ca^{2+} concentration **(319)**. ATP seems to promote stability in TRPV-ARDs which are capable of binding ATP **(177)**. Binding of phosphoinositide to the ARD of TRPV4 reduces the activity of the channel. PI(4,5)P2 exhibits the strongest level of binding to the ARD of TRPV4 and it is effective in regulating TRPV4 activity. Indeed, the activity of the channel is augmented by titration or hydrolysis of membrane PI(4,5)P2. Interestingly, mutations in TRPV4, which result in "gain-of-function"-type channelopathies, eliminate PI(4,5)P2-binding and sensitiy to PI(4,5)P2 on the part of the mutant TRPV4 **(395)**. TRPV4-^{121}AAWAA are mutant TRPV4 proteins which do not possess the phosphoinositide-binding site ^{121}KRWRK^{125} or the normal reaction to physiological stimuli. These mutants change the migration pattern of HEK293 cells in which they are expressed. Cells expressing these mutants also exhibit a diminution in the activity of calpain which appears to function in focal adhesion disassembly. Knock-down of TRPC1 (which is known to participate in cell migration) results in a similar cell migration pattern to that exhibited by HEK293 cells overexpressing TRPV4-^{121}AAWAA **(280)**.

Both high viscous load and hypotonicity activate TRPV4 *via* a phospholipase A_2 (PLA_2) dependent pathway, with the resulting production of EET being the principal mechanism for gating TRPV4. When only limited activation of PLA_2 is possible, these activators also use extracellular ATP-mediated production of PLC-IP_3 to regulate TRPV4 channel activity. IP_3 alone does not activate TRPV4, but, in epithelial ciliated cells and in cells heterologously expressing TRPV4, it has been found to be capable of sensitising TRPV4 to EET. The IP_3 receptor antagonist, xestospongin C, inhibits this effect. The IP_3 receptor engages in a physical interaction with TRPV4 **(116)**. IP_3 sensitises TRPV4 to stimulation by mechanical and osmotic stimuli as well as to direct EET stimulation. Binding of the inositol 1,4,5-trisphosphate receptor type 3 (IP_3R3) to TRPV4 is essential for IP_3-effected sensitisation of TRPV4. This binding site for IP_3R3 resides in a C-terminus region of TRPV4 which is coincident with a binding site for CaM **(130)**.

A 20-amino acid distal segment comprising residues 838-857 in the C-terminus area governs channel folding, maturation, and trafficking. Loss of this segment by mutant proteins results in their ending up stuck and unglycosylated in the endoplasmic reticulum with early degradation and ending of channel activity. This segment, comprising residues 838-857, is believed to adopt a specific conformation, with Gly849 and Pro851 being positioned at important locations. Causing the temperature to fall from 37°C to 30°C rescues TRPV4 channel function in folding-defective mutants. Interaction occurs between the C-terminus and the N-terminus as well as C-C terminus interaction **(222)**. Oligomerization is dependent on precise domains in both the N-terminus and the C-terminus of TRPV4. This process is preliminary to plasma membrane trafficking and occurs in the endoplasmic reticulum **(34).**

TRPV4 can become sensitised *via* both PKC and PKA pathways. Upon treatment with phorbol 12-myristate 13-acetate (PMA), TRPV4 is phosphorylated on Ser824 which activates PKC. When forskolin activates the PKA pathway, Ser824 is also phosphorylated. Influx of Ca^{2+} *via* TRPV4 is increased in both resting and stimulated cells when phosphorylation is mimicked at this site by the insertion of aspartic acid in lieu of Ser824 **(317)**. PKCa facilitates the activation of TRPV4 by acetylcholine in endothelial cells **(1).**

Interactions with other channels or channel sub-units may alter the behaviour of TRPV4. This may explain why the IV characteristics for TRPV4-dependent responses in mature differentiated cells may differ from those identified with homomeric TRPV4 channels in expression systems. Thus, the TRPV4 agonist GSK-1016709A activates a current in Muller glial cells that is blocked by the TRPV4 antagonist HC-067047 (332). This, however, does not show the classic outward rectification exhibited by homomeric TRPV4 (239, 433), being more akin to the stretch-sensitive voltage-independent cation current found in cultured Müller glial cells (325). This difference may reflect changes in the biophysical properties of homomeric TRPV4 channels physiologically expressed in Müller cells; but it appears more likely that it is due either to the formation of heteromeric TRPV4/TRPC1 channels or TRPV4/TRPP channels and/or to linearization of the current due to the co-expression AQPs (332).

4. Expression pattern of TRPV4

(i) Nervous system

Consistent with its involvement in a wide range of physiological functions, TRPV4 is disseminated throughout the body in a number of different cell types (**Table 1)**. In the central nervous system (CNS), functional TRPV4 channels, which are constitutively active at physiological temperatures, are found in hippocampal neurons **(294, 355)**. They are also expressed in rat cortical astroglia, being especially plentiful in astrocytic membranes found between the brain and extracerebral liquid spaces **(35)**, and in astrocytic "endfeet" that encase the blood vessels **(102)**. Astrocytes possess a TRPV4/Aquaporin-4 complex that is an essential component in the brain's volume homeostasis **(36)**. TRPV4 is found in the endothelium of cerebral arteries **(256)**, in magnocellular neurosecretory cells **(383)**, and in circumventricular organs in the CNS, which is a major location for osmotic sensing **(230)**. It is richly expressed in the apical membrane of epithelial cells of the choroid plexus where it funtions with anoctamin 1 in cell volume regulation **(396)**. TRPV4 aggregate in the cell body of immature neurons in the hippocampus rather than in post-synaptic locales. When the neurons are maturing, TRPV4 is found in dendrites and also accumulates in post-synaptic areas **(356)**. TRPV4 is expressed in cells of the immortalised neuroendocinre rat hypothalamic 4B cell line **(340).**

TRPV4 is found in peripheral sensory neurons where TRPV4 protein is transported distally along the axons in the direction of the peripheral nerve endings **(12, 233)**. Co-expression of TRPV4 and TRPV1 is found in a family of DRG neurons in rat and in their terminals located in the spinal dorsal horn **(53)**. TRPV4-expressing trigeminal ganglion sensory neurons **(72)** innervate the dura **(439)**, while DRG neurons expressing TRPV4 innervate the colon, skin and internal elastic membrane of the tongue **(44, 367, 388, 389)**. Osmosensitive sensory neurons expressing TRPV4 innervate the blood vessels of the liver and have their perikarya in thoracic DRG **(214)**. TRPV4 is also expressed in the *cauda equina* **(388)**, as well as in a range of other sensory and motor elements. These include both the inner and outer hair cells and spiral ganglion neurons in the cochlea as well as in sympathetic and parasympathetic nerve fibres, including those innervating the arrector pili smooth muscle in skin, sweat glands, intestine, and blood vessels **(88, 351, 392, 398)**. TRPV4 is found in the perikarya, axons and dendrites of retinal ganglion cells and at the optic nerve head in mouse, as well as in Muller glial cells **(333)**. **The satellite glial cells in a subpopulation of mouse DRG express functional TRPV4 (329).**

(ii) Other organ systems

TRPV4 is found in multiple organ systems outside the nervous system. TRPV4 channels are expressed in T cells where their activation results in Ca^{2+} ingress into those cells **(252).** TRPV4 is found in human corneal epithelial cells, retinal epithelial pigment cells, and endothelial cells of the eye **(266, 309, 478)**, in kidney, liver and heart **(2, 88, 159, 168, 180, 380, 388, 442)**, as well as in human odontoblast-like cells **(105),** and in the epithelial linings of both trachea and lung airways, serous cells of submucosal glands, and mononuclear cells **(88, 184)**. It is also found in the human oesophagus **(409)**, in stellate cells of the human pancreas **(470)**, and in Merkel cells from hamster buccal mucosa **(376)**. In mouse skin, TRPV4 is found in free nerve endings and at cutaneous mechanosensory terminals co-localised with neurofilament 200, including Meissner, Merkel, penicillate and intraepidermal terminals but not including hair follicle palisades **(389)**. TRPV4 has been suggested to act as a sensor/transducer of the mechanical stresses which are induced by flow in the collecting ducts of the kidney, where it is found throughout all of the collecting duct system, with functional ion channels demonstrated in principal cells and intercalated cells of split-opened

cortical collecting ducts and connecting tubules **(37, 388)**. Functioning TRPV4 is also demonstrated in the cell membrane of urothelial cells found in guinea-pig and mouse **(109, 451, 452)**, in the urothelium lining the renal pelvis, ureters, urinary bladder, and urethra of the rat **(39)**, while both human and mouse bladders express TRPV4 in bladder urotehlial cell membranes near adherence junctions, with these ion channels being molecularly connected to those junctions **(183)**. TRPV4 is found in primary nasal epithelial cells in humans where it regulates Ca^{2+} levels in epithelial cells and ciliary beat frequency, suggesting a role in mucociliary clearance and airway protection **(6)**. Laryngeal epithelium also expresses TRPV4 **(153)**, as does mouse olfactory epithelium **(3)**, and rat oral mucous membrane (**283)**. Vascular endothelial cells, including human brain capillary endothelial cells, also express TRPV4, as do vascular smooth muscle cells **(80, 103, 104, 160, 257, 435)**, human synoviocytes **(180)**, articular chondrocytes **(304)**, the guinea-pig and human endolymphatic sac **(393)**, and the acinar cells of the mouse submandibular gland **(472)**. TRPV4 is found in the endometrium and also in the myometrium of the uterus of the pregnant and the non-pregnant mouse. Agonist-induced activation of TRPV4 increases myometrial contractions whether the animals are pregnant or not. The contractions thereby induced can be blocked by a TRPV4 antagonist leading to the suggestion that the myometrial contractitlity induced by the activation of TRPV4 by endogenous ligands can be reduced by blocking TRPV4 where required in pre-term labour **(366)**. Human endometrial stromal cells in culture expresss functional TRPV4 in both the cytoplasm and plasma membrane **(85)**. TRPV4 is also expressed in human hepatoblastoma (HepG2) cells **(422)**

TRPV4 may also be present in skeletal muscle since exposure to hypo-osmotic solutions increases the opening of mechanosensitive channels in skeletal muscle while recordings from skeletal muscle taken from TRPV4 knockout mice fail to show mechanosensitive activation of that channel **(169)**. Functional TRPV4 are particularly plentiful in human pre-adipocytes (i.e., adipocyte precursor cells) where they are involved in adipogenesis *via* phosphorylation of Akt kinase **(66)**. **Functional TRPV4 receptors are found in human sebocytes where they mediate the lipostatic and antiproliferative effects of cannabidiol (305). TRPV4 is also found in monocytes and in monocyte-derived dendritic cells (Szollosi et al., 2013), as well as in Langerhans' cells (385) , and in human pluripotent stem cell-derived cardiomyocytes in culture (326).**

5. Activators of TRPV4

(i) Mechanical deformation and osmotic stimuli

The sensitivity of a mouse's tail to pressure is markedly reduced when the TRPV4 gene has been disrupted. Both the threshold to noxious stimulation by pressure and the conduction speed of myelinated nerves in response to pressure are also reduced **(388)**. This suggests that TRPV4 is involved in the transduction of mechanical pressure applied to tissues when that pressure goes beyond light touch. However, TRPV4 is also a more subtle transducer of pressure at the cellular level in that it responds to extracellular osmotic pressure. Indeed, TRPV4 was originally known as vanilloid receptor-related osmotically activated channel (VR-OAC) because it was first characterised as a receptor which responds to changes in extracellular osmolarity **(Figure 6)**. Thus, Strotmann and colleagues (2003) found that, in isotonic media, TRPV4 exhibits spontaneous activation. However, decreases in extracellular osmolarity result in rapid TRPV4 activation, while increases in extracellular osmolarity inhibit activation of TRPV4 **(380)**. The role of extracellular hypotonicity as an activator of TRPV4 is well-established, but the effect of extracellular hypertonicity is less definite although this can, in some circumstances, result in TRPV4 activation. Levine's group have shown that exposure to hypotonic solution activates 54% of C-fibers in rat and that this effect is enhanced by PGE2 **(12)**. They have also shown that TRPV4 mediates pain induced by mild hypertonicity in rat where intradermal injection of 2 per cent saline solution in the animal's hind-paw induces a concentration-dependent pain-related behaviour, flinching **(11)**. Observations on pressurised arterioles also support the concept that TRPV4 channels are sensitive to mechanical loading, since reductions in intravascular pressure activate endothelial TRPV4 channels **(22)**.

There is no evidence yet that TRPV4, across its multiple applications, acts as a sensory transducer in respect of mechanical force applied to the cell membrane. It has yet to be established that activation of TRPV4 as a result of force applied to the cell membrane can produce the graded response typical of sensory transducers. Hence, the use of the descriptive terms "osmosensor" and "mechanosensor" in relation to TRPV4 has the potential to mislead. On the other hand, it is eminently clear that TRPV4 does respond, in several organ tissue

types at least, to the application of force to the cell membrane – whether that mechanical force is generated by hypotonicity, hypertonicity or trauma – to induce physiological effects. So it is certainly correct to describe TRPV4 as being "mechanosensitive." However, it is not established how the mechanical pressure brings about the gating of TRPV4 in those contexts where the ion channel is activated by mechanical force to induce physiological effects. That mechanical pressure when applied to the cell memberane will typically result in (some) deformation of the cell membrane seems obvious. The concept of deformation of the cell membrane embraces that of "cell stretch," although such deformation may also be envisaged without cell stretch. Certainly, it is now clear that "cell stretch" can activate TRPV4 in multiple organ tissue types. Thus, in urothelial cells from TRPV4 null mice, the increase in $[Ca^{2+}]_i$ evoked by stretch applied in a cell-stretch system is significantly reduced compared to that evoked in wild-type cells **(272)**, while TRPV4 in capillary endothelial cells adherent to flexible extracellular matrix substrates are activated when cyclically stretched **(400)**. Again, rat TRPV4 responds to pipette suctions that stretch the excised membrane patches **(239)**, while "shear stress" evoked by flow, which inevitably involves stretch, activates TRPV4 in vascular endothelium **(263)**. Finally, Merkel cells from hamster buccal mucosa express TRPV4 which are activated by membrane stretch **(376)**.

The further (controversial) question remains, however, as to what constitutes the mechanism of action of such cell stretch, or other deformation of the membrane, which induces the activation of TRPV4? There are several possible mechanisms of activation of TRPV4 in this context. First, TRPV4 may respond directly to the effect of mechanical deformation of the membrane. Variations in tonicity of the extracellular fluid may result in activation of TRPV4 to the extent that they alter the tension in the cell membrane. On this view, mechanical deformation of the membrane – whether secondary to hypotonicity or to a direct mechanical pressure impinging on the cell membrane – is the ultimate activator of TRPV4 by mechanical pressure. Liedtke (2005), however, has correctly observed that a change in cell volume does not necessarily lead to a significant alteration in membrane tension where the membrane is loose as a result of its engagement in some other function, such as process formation **(228)**. Loukin and colleagues (2010) expressed rat TRPV4 in Xenopus oocytes and repeatedly examined more than 200 excised patches bathed in a simple buffer. They found that TRPV4 can be activated by tens of mm Hg pipette suctions with open probability rising with suction even in the presence of relevant enzyme inhibitors designed to eliminate any effect of enzymes brought into existence by the suction pressure

(239). Janssen and co-workers (2011) found TRPV4 to be present in human bladder urothelial cell membranes near adherence junctions and also identified the existence of a molecular connection of the ion channel to alpha-catenin (a component of the adherence junction that ties E-cadherin to the actin-microfilament network). They suggested that the binding of TRPV4 to a rigid intracellular and intercellular structural network is consistent with the hypothesis that TRPV4 is activated by bladder stretch **(183)**. **TRPV4 has been shown to function in the cyclic stretch-induced realignment of human pluripotent stem cell-derived cardiomyocytes in culture. Longitudinal stretch induces an increase in $[Ca^{2+}]_i$ which is inhibited by antagonists of TRPV4. Moreover, 2 hours of uniaxial cyclic stretch results in a realignment of these cells in the direction transverse to the direction of stretch – a result which is also inhibited by antagonists of TRPV4 (326).** The evidence of TRPV4 interaction with the cytoskeleton **(142)** supports the concept of mechanical deformation of the cell membrane *per se* being capable of activating TRPV4. Interestingly, the stretch-activated channels (SACs) TRPC1 and TRPC6 are also activated by mechanical and hypotonic stimuli and these ion channels are often found expressed with TRPV4 in DRG neurons. Spinal intrathecal administration of oligodeoxynucleotides antisense to TRPC1 and TRPC6, or to TRPV4, reverses the hyperalgesia to mechanical and hypotonic stimuli induced by inflammatory mediators without affecting baseline mechanical nociceptive threshold, and it has been proposed that TRPC1 and TRPC6 channels cooperate with TRPV4 channels to mediate mechanical hyperalgesia and primary afferent nociceptor sensitisation **(7, 290)**. Although the view that cell stretch does not gate TRPV4 by deformation of the membrane *simpliciter* appears to be in the ascendency at the moment, there are two very plausible mechanisms whereby mechanical force *could* gate the TRPV4 channel **(315)**. Thus, the application of mechanical force to the cell membrane can effect an alteration in the forces active within the lipid membrane which effect conformational changes within the ion channel and result in channel gating because of energy differences created between membrane tension in the open and closed conformations, respectively. In this context, the lipid-bilayer directly effects the opening of TRPV4 when appropriate mechanical force is applied to the membrane. An alternative theory postulates that the opening of TRPV4 is mediated by the response to mechanical force applied to cell membrane structures attached, or tethered, to the ion channel. These structures include accessory proteins, the cytoskeleton or even the extracellular matrix. The mechanical force is transmitted *via* these structures to effect an alteration in the conformation of the channel which results in gating **(46, 208, 315**). In the case of the TRAAK and TREK1 ion channels – which are mechanosensitive K^+

channels with a two-pore domain – gating has recently been shown to be effected directly *via* the lipid bilayer as "membrane-tension-gated" channels **(46)**. The manner in which the tension of the lipid bilayer is employed to regulate the gating and mechanosensitivity of the TRAAK ion channel has also been elucidated. In the non-conductive state, the passage of ions is physically prevented by a lipid acyl chain entering the channel pore *via* a 5 Å-wide lateral opening in the inner leaflet of the cell membrane. In the conductive state, ion entry is allowed by the action of a transmembrane helix (TM4) which rotates about a central hinge to seal the intramembrane opening and preclude the lipid block of the pore. An increase in cross-sectional area of up to 2.7 nm^2 in the conductive state is believed to result in a membrane-tension-dependent difference in energy levels between conformations that induces force activation of the chanel **(45)**. These findings support the plausibility of TRPV4 being gated directly by the application of force to the cell membrane – although, given its range of activators, it could not be unexpected that TRPV4 should have more than a a single mechanism of activation **(426)**.

A further putative mechanism of action which is consistent with the mechanosensitivity of TRPV4 is that membrane deformation may cause the release and activation of membrane products which, in turn, effect gating of TRPV4. Thus, cell swelling has been described as activating TRPV4 by means of PLA_2-dependent formation of arachidonic acid and its subsequent metabolization to 5',6'-EET by means of a cytochrome p450 epoxygenase-dependent pathway **(434)**. The implication of these latter findings is that it is not necessarily the abnormal mechanical pressure, as such, that proximately gates TRPV4 but rather the chemical signals generated in response to changes in cell stretch or other deformation of the cell membrane. Both high viscous loading and hypotonicity have been said to employ the activation of phospholipase A_2 (PLA_2) and the resulting production of EET as the principal mechanism for gating TRPV4 in native ciliated epithelial cells. When only limited activation of PLA_2 is possible, these stimuli employ extracellular ATP-mediated activation of IP_3 to gate TRPV4. IP_3 is not itself an agonist of TRPV4. However, it has been found to sensitise TRPV4 to EET in epithelial ciliated cells and in cells which heterologously express TRPV4. The IP_3 receptor antagonist, xestospongin C, inhibits this result. A physical interaction appears to occur between TRPV4 and IP_3 receptor 3 (**116)**. IP_3-mediated sensitisation of TRPV4 requires IP_3 receptor binding to a C-terminus domain of TRPV4 which coincides with the location of a CaM binding site **(130)**.

A third possibility is that alterations in extracellular tonicity *per se* (induced by mechanical pressure or otherwise) activate intracellular proteins independently of affecting membrane tension, and that this in turn leads to gating of TRPV4. Osmotic stimulation can result in the activation of a succession of intracellular phosphorylation/dephosphorylation signalling processes **(42, 43, 228)**.

Whatever the underlying mechanism of activation, TRPV4 is undoubtedly important in the homeostatic control of cell volume in the face of an osmotic challenge. When the extracellular fluid is hypotonic (i.e., at lower osmotic pressure than that found within the cell), the cell swells due to osmosis. Swelling is followed by RVD, which allows the cell to regain its former volume notwithstanding that the extracellular fluid remains hypotonic. Studies in a human keratinocyte cell line indicate that TRPV4 directly participates in RVD **(33)**. Hypotonicity increases the association and surface expression of the water permeable channel AQP5 and TRPV4 in salivary gland cells. Both channel association and trafficking, as well as RVD, are reduced by actin depolymerization. Activation of TRPV4 by hypotonicity does not depend on cell swelling itself, but rather on AQP5. TRPV4 and AQP5 are together believed to control RVD **(234)**. Hypotonic stress results in phosphorylation of residue Tyr253 of TRPV4 by a Src family tyrosine kinase. This residue is essential for channel function in this context as a point mutation of Tyr253 precludes the activation of TRPV4 by hypotonicity **(450)**. Extracellular hypotonic solution induces stretch of the plasma membrane of mouse odontoblast lineage cells which results in TRPV1-, TRPV2-, and TRPV4-, mediated Ca^{2+} influx and inward currents. The increase in intracellular-free Ca^{2+} concentration is extruded by Na^{+}-Ca^{2+} exchangers **(339)**. Although TRPV4 directs behavioural responses to osmotic and mechanical stimuli **(230)**, base-line sense of touch and non-noxious/non-nociceptive light touch are not mediated by TRPV4, i.e., TRPV4 only detects “a nociceptive level of mechanical stimuli” **(389)**.

TRPV4 may not always promote RVD, as TRPV4 inhibition actually decreases swelling in response to hypotonic challenge in both Müller glial cells and ganglion cells (332). This detailed study also identified key differences between the TRPV4-mediated response to extracellular hypotonicity in these two cell types, with distinct kinetics and activation pathways. Hypotonic activation of TRPV4 in retinal glial cells, but not in retinal neurones (ganglion cells), was dependent on a phospholipase A2/cytochrome p450 pathway. TRPV4 mediated increases in $[Ca^{2+}]_i$ were seen in Muller glial cells when

extracellular tonicity was reduced to less than 70% of normal, with a maximal response at or below 50% (332).

(ii) Heat

TRPV4 can be activated by heat. When the temperature is increased above 25ºC. currents are activated and $[Ca^{2+}]_i$ is increased in both HEK293 cells transfected with TRPV4 and in endothelial cells of mouse aorta. However, after removal of the three N-terminus ankyrin binding domains of TRPV4, heat fails to activate transfected TRPV4 in HEK293 cells **(435)**. It has subsequently been found that this failure in activation is due to a defect in TRPV4 expression at the cell membrane in HEK293 cells and, moreover, that TRPV4 loses its sensitivity to stimulation by heat when expressed in excised cell membrane patches **(76, 435)**. TRPV4 generates large inward currents in Xenopus oocytes, as well as inward currents and Ca^{2+} influx in HEK293 cells, at temperatures which are less than those needed to activate TRPV1. The TRPV4 response to heat is increased in hypo-osmotic solutions and reduced in hyperosmotic solutions. The threshold for activation of TRPV4 by heat varies with the context in which these ion channels are expressed. Thus, activation of TRPV4 is found at temperatures >27˚ C in oocytes, while in HEK293 cells the threshold for activation is ~34˚ C. Continued stimulation of TRPV4 with heat above these thresholds causes the channel to desensitise. However, once TRPV4-expressing HEK293 cells have been acclimated at 37˚ C, TRPV4 has the capacity to respond to additional increases in temperature **(151)**. This implies that this ion channel is constitutively active at normal body temperature. Moreover, heat, at 37°C, increases the efficacy of other stimuli in activating TRPV4 **(129)**. Heat (and phorbol esters) activate TRPV4 by means of a pathway in which an aromatic residue at the N-terminus of the third transmembrane domain plays an essential role and which is independent of PLA_2 and cytochrome P450 epoxygenase **(426)**. The heat-evoked currents observed in the majority of mouse keratinocytes appear to involve TRPV4 and exhibit an activation threshold of ~32 °C. **(76, 77)**.

Behaviourally, there is no agreement on the role of TRPV4 as a contributor to thermal responsiveness *in vivo,* although it does not appear to be a critical regulator of behaviour in response to heat. Thus, TRPV4 null mice have been found by Lee and colleagues to select warmer floor temperatures than wild-type mice and to respond with extended withdrawal latencies during acute application of heat to the tail, although no distinction is found between

normal and knockout mice as regards circadian body temperature fluctuations and thermoregulation in a warm environment **(217)**. Todaka and colleagues found that wild type and TRPV4 null mice show the same latency of escape from 35-50^{o}C hot-plates; but mice lacking TRPV4 which suffer hyperalgesia as a result of carrageenan injection take a significantly longer time to leave hot-plates at 35-45^{o}C than do wild-type animals **(404)**. On the other hand, Huang and co-workers found that TRPV4 knockout mice show thermal preference behaviour similar to wild-type animals on a thermal gradient, and little or no change in acute heat nociception or inflammatory heat hyperalgesia, even when TRPV1 is inhibited. These authors conclude that the probability is that the involvement of TRPV4 in sensing harmless and noxious heat sensations is limited and strain-dependent and that one must look elsewhere to find the mechanism(s) which act as thermosensor **(175, 423)**. It has been suggested that thermosensation in mammals depends almost totally on two populations of sensory neurons which are "differentially tuned" to detect a range of temperature sensations (cold, cool, warm and hot). Thus, neurons that express TRPV1 respond to high temperature and mediate the behavioural responses in mouse in the temperature range of 40^{o} C to 50^{o} C. Neurons that express TRPM8 are responsible for cold aversion. More extreme cold and heat cause the activation of further populations of sensory neurons, including those which express Mrgprd **(321)**.

Nicotinic acid, which inhibits the activity of TRPV4, also increases the ion channel's threshold for activation by heat **(242)**.

Activation of peripheral TRPV4 channels by topical treatment of the skin of rat with a TRPV4 agonist, RN-1747, results in hypothermia caused by activation of peripheral TRPV4 channels. Blocking TRPV4 by intravenous admnistration of HC-067047, a TRPV4 antagonist, increases core body temperature when the temperature of the surrounding environment is at 26° C or 30° C, but does not do so at 22° C or 32° C. The hyperthermia induced at 26° C develops with a concomitant increase in the animal's consumption of oxygen, while pharmacological activation of TRPV4 results in an increase in tail heat loss, suggesting the involvement of TRPV4 in mediating these autonomic functions in rat **(418)**.

(iii) pH

TRPV4 expressed *in vitro* in Chinese hamster ovary cells has been reported to be opened by low pH or citrate, and mice which lack the TRPV4 receptor exhibit a substantially diminished response to acids (**388)**. On the other hand, acid (pH 5.0) has been found to inhibit Ca^{2+} influx *via* TRPV4 in mouse oesophageal epithelial cells, leading to the suggestion that the exposure to gastric acid of cells which express TRPV4 – as occurs in cases of gastro-oesophageal reflux disease – may adversely affect cell function and aggravate the sufferer's condition **(359)**.

(iv) PLA_2-mediated metabolites of arachidonic acid

The endogenous cannabinoid neurotransmitter, anandamide, also known as ***N***-arachidonoylethanolamine, and its metabolite, arachidonic acid, gate TRPV4 channels indirectly *via* the formation of epoxyeicosatrienoic acids (EETs) in a cytochrome P450 epoxygenase-dependent pathway. Arachidonic acid induces Ca^{2+} entry into coronary endothelial cells *via* TRPV4. The initiation of this Ca^{2+} response involves, *inter alia*, membrane hyperpolarisation, phosphorylation of TRPV4 *via* PKA, and the action of arachidonic acid on gating of TRPV4 (Zheng et al., 2013) **(480)**. TRPV4 is activated in a membrane-delimited fashion by the application of 5',6'EET at below micromolar concentrations. The like application 5',6'EET occasions Ca^{2+} influx through TRPV4 channels in vascular endothelial cells ((**424, 434)**; **Figure 6**). Cannabidiol activates TRPV4 in cultured human sebocytes and human skin organ culture **(305)**.

The *N*-acyl amides are a family of endogenous lipids which are structurally similar to anandamide and may provide activators of TRPV4 **(327)**. Indeed, *N*-arachidonoyl taurine has been shown to activate TRPV4 as well as TRPV1, although it fails to activate the CB receptors **(334)**.

(v) Inflammation

Inflammation increases interstitial fluid volume and tonicity, with local accumulations of cellular debris and other products of the inflammatory response. This creates abnormal mechanical pressures on the plasma membranes of local cells and *inflammagens* are inevitably introduced into the immediate environment of these cells **(440)**. In addition, TRPV4 is capable of being gated in response to one, or more, products of the local

inflammatory response to injury. For example, increased tissue temperature and, and in certain instances, reduced pH have been found to activate TRPV4 *(supra)*; and these are also components of the local inflammatory response. Exposure to hypotonic solution or administration of 4α-PDD results in the release of neuropeptides in the urinary bladder and airways of mice. Oedema formation, as well as recruitment of granulocytes, results from intraplantar injection of the same TRPV4 activators. Moreover, knockdown of the TRPV4 gene, or its deletion, reduces the oedema formation as well as granulocyte infiltration which results from intraplantar formalin injection in the mouse **(413)**. The combination of an inflammatory mediator with a mild hypertonic stimulus activates TRPV4 to induce behaviour attributable to pain in animals **(11)**. Ablation of TRPV4 in rat eliminates the increase in the pain-induced paw-withdrawal reflex which is found on exposure to mechanical or hypotonic stimulation after intradermal injection of carrageenan or a "soup" of inflammagens. Consistent with the foregoing, intradermal injection of inflammatory soup fails to elicit significant osmotic or mechanical hyperalgesia in TRPV4 knockout mice **(8)**. When prostaglandin E2 and serotonin is injected into the mechanical receptive fields of C-fibres in wild-type and TRPV4 knock-out mice, the wild-type animals show both an increase in the number of C-fibres which respond to hypotonic stimuli and an increase in the extent of that response when compared with mice which lack TRPV4. Moreover, evidence of receptor sensitisation, in the form of more spontaneous activity and reduced mechanical threshold, is only found in C-fibres from the wild-type animals **(70)**. When activated, the TRPV4 found in epithelial cells of the intestine results in chemokine release and colitis. The development of colitis is associated with an increase in colonic permeability **(82)**. Increased expression of TRPV4 mRNA is found in the gastrointestinal tract of patients suffering from inflammatory bowel diseases. Moreover, in an animal model of colitis, specific inhibition of TRPV4 reduces colitis and intestinal inflammation-induced pain **(119)**. TRPV4 is sensitised to agonist challenge by prior exposure to serotonin or histamine. Such exposure also results in increased expression of TRPV4 in the plasma membrane of sensory neurons in the colon **(59)**. Protease-activated receptor 2 (PAR_2), when activated, sensitises TRPV4 to result in mechanical hyperalgesia, while removal of TRPV4 prevents mechanical hyperalgesia induced by agonist activation of PAR_2 and sensitisation **(147)**. Activation of PAR_2 induces only a transient increase in $[Ca^{2+}]_i$ in cells which do not express TRPV4. However, in the presence of TRPV4, activation of PAR_2 leads to a sustained increase in $[Ca^{2+}]_i$. This activation of TRPV4 by PAR_2 is dependent on a key tyrosine residue (Tyr-110) without which PAR_2 is unable to activate TRPV4 **(322)**. Cathepsin S is another product of the

inflammatory response which can activate PAR_2 which then activates TRPV4. Its injection in mouse results in inflammation and hyperalgesia which is reduced by deleting either PAR_2 or TRPV4 or by inhibiting adenylyl cyclase **(476)**. TRPV4 functions in the development of inflammation in adipose tissue in mice occasioned by inflammatory insult, although this may be ameliorated by a prior regime of exercise. Exercise-trained mice show a reduction of ~50% in the genes for IL-6, SOCS3 and tumour necrosis factor alpha (TNF-α) in epididymal adipose tissue induced by acute inflammatory challenge, as well as a reduction in the increases in plasma IL-6 found in non-trained animals. These results are associated with reduction in the expression of TRPV4 **(56)**. Expression of TRPV4 is increased in burn scars which exhibit post-burn pruritus **(456).**

(vi) Dimethylallyl pyrophosphate (DMAPP)

Dimethylallyl pyrophosphate (DMAPP) is an endogenous activator of TRPV4. TRPV4 is activated by micromolar concentrations of DMAPP in transfected HEK cells, in cultured sensory neurons and in keratinocytes. DMAPP induces acute inflammation and noxious mechanical hypersensitivity in a TRPV4-dependent manner It also acutely elicits flinching on intraplantar injection *in vivo* in mice which is prevented by pre-treatment with TRPV4-blockers **(27)**.

(vii) Phorbol derivatives: 4α-PDD and PMA

Phorbol is an organic compound obtained from croton oil which comes from the seeds of croton plants. Phorbol derivatives are agonists at the TRPV4 ion channel and are capable of directly gating TRPV4 **(432)**. The phorbol ester, 4α-PDD, is the most specific known agonist of TRPV4 and one of its most effective activators (**Figures 2(B) & 8(B)**). It is widely employed experimentally, although its exclusivity for TRPV4 as its target receptor has been put in issue by the claim that it can activate cultured mouse DRG neurons independently of TRPV4 **(13)**. Phorbol 12-myristate 13-acetate (PMA) is a powerful activator of PKC but is some 50 times less effective than 4α-PDD in effecting an increase in $[Ca^{2+}]_i$ in transfected TRPV4-expressing cells, notwithstanding its structural similarity to 4α-PDD **(432)**. Exposure to 4α-PDD induces a monophasic or biphasic increase in $[Ca^{2+}]_i$. The PMA-induced increase in $[Ca^{2+}]_i$ is eliminated where a PKC-specific inhibitor has been

introduced before exposure to PMA. However, the same inhibitor has no effect on the increase in $[Ca^{2+}]_i$ caused by 4α-PDD, which proves the involvement of mechanisms which are, respectively, dependent and independent of PKC in the activation of TRPV4 by phorbol esters **(448).** Gating by 4α-PDD of TRPV4 channels depends on the presence of both extracellular and intracellular Ca^{2+}. Mutations of single amino acid residues in the sixth transmembrane domain and in the C-terminus of TRPV4 affect this gating **(433)**. Mutations of two hydrophobic residues in the central part of transmembrane segment 4 (Leu584 and Trp586) result in a substantial loss of sensitivity of TRPV4 to 4α-PDD, bisandrographolide A, and heat. However, these mutations do not affect the response of TRPV4 to arachidonic acid, 5,6-EET, or cell swelling. In contrast, gating of TRPV4 by multiple activating stimuli is affected by mutations of two residues in the C-terminus part of transmembrane segment 4 (Tyr591 and Arg594). This implies that these mutations impact on channel gating rather than with the channel's activators (**425)**. Ligand-binding affinity for TRPV4 has been proposed to be determined by the length of the fatty acid moiety **(425)**. Phorbol esters and heat rely on an activation pathway for TRPV4 which is independent of both PLA_2 and cytochrome p450 epoxygenase. This pathway depends on an aromatic residue at the N-terminus of the third transmembrane domain **(426)**. 4α-phorbol esters bind in a loop found in the TM3-TM4 domain of TRPV4. This binding site is similar to that of capsaicin to TRPV1. Both the ester decoration of ring C as well as the A,B ring junction have been found to be essential for activity. Ring C has lipophilic ester groups which are important in directing the diterpenoid core into the pocket of the ligand-binding site. The A,B ring junction is responsible for the activation of TRPV4 being dependent on Ca^{2+}. 4α-PDH exhibits a potency in activating TRPV4 which is almost five times greater than that of 4α-PDD **(201)**.

(viii) Other plant extracts

Bisandrographolide A (BAA) is to TRPV4 what capsaicin is to TRPV1, i.e., a potent plant-derived agonist exclusive to its target receptor. BAA is a compound isolated from an extract of the plant Andrographis Paniculata which can activate TRPV4 with an EC(50) of 790-950 nM. However, it does not activate, or block activation of, TRPV1, TRPV2, or TRPV3 channels. BAA activates a large TRPV4-like current in immortalised mouse keratinocytes (308 cells) which are known to possess TRPV4. It also generates TRPV4 currents in outside-out patches from HEK293T cells which overexpress TRPV4 cDNA **(369, 425)**.

Apigenin is a plant-derived flavone which is also an agonist at the TRPV4 receptor. Apigenin gates TRPV4 dose-dependently in HEK cells which over-express this receptor and in native endothelial cells. It acts on TRPV4 in endothelial cells of small mesenteric arteries in rat to induce endothelium-derived hyperpolarizing factor (EDHF)-mediated dilation **(246)**.

Eugenol is a liquid which can be obtained from the oil of cloves and from that of several other plants. It is used as an analgesic in dentistry. It is a vanilloid molecule which activates TRPV4 ion channels expressed in the endothelial cells of rat mesenteric arteries to dilate those blood vessels and reduce the systemic blood pressure **(316).**

Plant cannabinoids, like cannabidivarin and tetrahydrocannabivarin, exert agonist effects at TRPV4 to increase $[Ca^{2+}]_i$ with an efficacy and potency in the moderate to high range. Plant cannabinoids also act as potent desensitizing agents of TRPV4 **(92)**. Cannabidiol activates TRPV4 in cultured human sebocytes and human skin organ culture **(305).**

(ix) Sensitisation of TRPV4 by phosphorylation

Src family tyrosine kinases (SFKs) mediate tyrosine phosphorylation of TRPV4 **(437, 450)**. Tyr110 and Tyr805 are SFK-induced phosphorylation sites found in the N-terminus and C-terminus cytosolic tails in TRPV4, respectively. Phosphorylation of the N- terminus tyrosine by SFKs is effected before the activation of TRPV4 which suggests that tyrosine phosphorylation sensitises TRPV4 rather than activates it. The fact that reactive oxygen species, which are involved in mediating inflammatory pain, potently up-regulate the phosphorylation of TRPV4 when SFKs are present is consistent with a role for TRPV4 in mediating inflammatory pain **(4437)**. The phosphorylation of specific sites of the TRPV4 ion channel by PKC and PKA increases the activation of the ion channel. Such phosphorylation requires the assembly by AKAP79 of PKC and PKA into a signaling complex with TRPV4 **(112)**. PMA induces the phosphorylation of TRPV4 on Ser824 which is found in the C-terminus cytosolic tail of the ion channel. PKC, and perhaps also PKA, phosphorylate this ion channel at Ser824 resulting in its sensitisation **(317)**. In addition, phosphorylation at Ser824 is necessary for the interaction of TRPV4 with F-actin, as well as the appropriate subcellular localisation of TRPV4. Phosphorylation at this site not only promotes the activity

of the channel but also results in protein stability as well as expansion of the cell membrane **(361)**. PKC and AKAP150 (the anchoring protein for PKC) are necessary for TRPV4 activation mediated by muscarinic receptors **(375)**.

(x) Involvement of Protein Kinase G in TRPV4-mediated effects

Protein Kinase G (PKG) – also known as cGMP-dependent protein kinase – is a serine/threonine specific protein kinase which phosphorylates a variety of targets relevant to TRPV4. Vascular smooth muscle hyperpolarization and relaxation in coronary arteries induced by 11,12-EET is inhibited by NO. This depends on a PKG-pathway which phosphorylates TRPC1 in the TRPV4-TRPC1-KCa1.1 complex at Ser172 and Thr313 **(471)**. Flow-mediated Ca^{2+} influx into M1-cortical collecting duct cells *via* heteromeric TRPV4-P2 channels is inhibited by the actions of cGMP and PKG on these ion channels, through phosphorylation of $TRPP2^{T719A}$ and $TRPP2^{S827A}$ **(99)**. The flow-induced ingress of Ca^{2+} into vascular endothelial cells *via* the TRPV4-C1 complex is also negatively controlled by PKG **(247)**. It appears that increases in $[Ca^{2+}]_i$ resulting from activation of TRPV4 occasioned by hypo-osmotic stress of outer hair cells in the cochlea of the guinea pig result in the production of NO which, in turn, inhibits $[Ca^{2+}]_i$ increase by a NO-cGMP-PKG pathway feedback mechanism **(397)**. Similarly, increased intravascular pressure in pulmonary microvessels induces TRPV4-mediated endothelial Ca^{2+}-entry in endothelial cells, which both increases vascular permeability and activates a NO-dependent negative-feedback mechanism limiting the rises in both $[Ca^{2+}]_i$ and filtration coefficient **(458)**. Inhibition of PKG reduces thermal hyperalgesia (evidenced by a decrease in pain-related behaviour on exposure to heat) in rats suffering from chronic compression of the DRG, suggesting that a TRPV4-NO-cGMP-PKG pathway may contribute to thermal hyperalgesia resulting from this type of injury **(94)**.

(xi) Effect of serotonin or histamine on TRPV4 activation

There is some evidence that serotonin can activate TRPV4 in pulmonary arterial smooth muscle cells in rat **(101)**. TRPV4 is sensitised to agonist challenge by prior exposure to

serotonin or histamine. Such exposure also results in increased expression of TRPV4 in the plasma membrane of sensory neurons in the colon **(59)**.

(xii) UVB radiation

UVB radiation activates epidermal TRPV4 in rat which, in turn, results in an increase in the expression of endothelin-1 which mediates pain. TRPV4 is therefore implicated in the pain and tissue damage caused by sunburn **(276)**.

6. TRPV4 as a molecular integrator of diverse stimuli

TRPV4 is a polymodal ion channel, activated by a range of diverse stimuli. Simultaneously applied stimuli of quite different natures may interact so that their effect, as mediated by TRPV4, is not necessarily determined by the sum of the responses to the same stimuli when applied individually. Thus, exposure of cells transfected with TRPV4 to hypotonic solution at room temperature, or to 4α-PDD, induces minor channel activation, while PMA and shear stress have little, or no, effect on activation of TRPV4. However, at physiological body temperature (37˚C), TRPV4 is readily activated by all agonist stimuli **(129)**. This response is typical of many TRP channels and denominates them as efficient integrators of numerous diverse stimuli arising from various independent inputs. The resulting channel behaviour is characteristic of signalling patterns seen in many physiological and pathophysiological conditions **(157)**.

7. Antagonists of TRPV4

Known agonists and antagonists of TRPV4 are shown in **Table 2.** A selective antagonist of TRPV4 has yet to be definitively identified, but HC-067047 is believed to be selective for TRPV4. Ruthenium red is commonly used in experiments as a TRPV4 channel blocker that reversibly inhibits inward but not outward TRPV4 currents **(432)**, but it is not selective for TRPV4. Like gadolinium and lanthanum, ruthenium red inhibits TRP channels generally **(220, 296)**. Citral is sometimes mentioned as an antagonist of TRPV4, but it has multiple actions, activating TRPV1, TRPV3, TRPM8, and TRPA1 in primary afferents and producing a persistent blocking of TRPV1, TRPV2, TRPV3, and TRPM8 and temporary inhibition of

TRPV4 and TRPA1 **(379)**. **The plant alkaloid, berberine, found in various Chinese herbal remedies, inhibits TRPV4 (429).** Several small molecules have been suggested to function in the relief of various conditions **(166)**, most strikingly in relation to the resolution of pulmonary oedema resulting from heart failure where GSK2193874 has been shown to function as an orally effective TRPV4 antagonist which blocks the influx of Ca^{2+} through recombinant TRPV4 ion channels as well as native TRPV4 currents in endothelium **(176, 401)**. However, none have been shown to be selective for TRPV4. Butamben blocks TRPV4 at micromolar concentrations, and it reduces acute animal pain behaviours in a TRPA1- or TRPV4-dependent manner **(26)**. Resolvin D1 (RvD1) is an endogenous anti-inflammatory lipid molecule which reduces the activation of TRPV4 (and also TRPV3 and TRPA1) at nanomolar and micromolar concentrations. It can also produce an analgesic effect in the context of inflammatory pain **(28)**. Given the problems of collateral injury presented even by a selective TRPV4 antagonist when administered for a therapeutic purpose, a non-specific channel inhibitor which coincidentally blocks other TRP channels in addition to its TRPV4 target, presents extraordinary risks of complications arising out of its use in drug therapy. The antagonist RN-1734 completely inhibits both ligand-induced, and hypotonicity-induced, activation of TRPV4, without affecting the activity of other TRP channels, including TRPV1, TRPV3 and TRPM8, making it a useful tool for laboratory use **(417).** However, as with TRPV1, the difficulty in developing an antagonist of TRPV4 for therapeutic purposes resides in identifying a potent, selective and bioavailable small molecule which can target the TRPV4 channels of interest while preserving the function of TRPV4 channels necessary for non-pathological physiology. HC-067047 is a TRPV4 antagonist that has been found to improve working bladder capacity and to reduce frequency of micturition in rats and wild-type mice with cystitis. At the same time, it does not have an effect on bladder function in mice which lack TRPV4, suggesting that it may have a selective beneficial effect in this context **(110)**.

8. TRPV4 knockout mice

Two different “knockout” mouse models of TRPV4 have been developed **(230, 270)**. So where there is a conflict between the results of similar experiments derived from the responses of TRPV4 null mice, the initial question to be considered is whether that conflict may be capable of explanation by different TRPV4 knockout models having been used in the

respective experiments. An additional complication concerns the reliability of TRPV4 null mice as an indicator of TRPV4 function. In the case of animals which have been genetically engineered, there is always a question as to whether loss of the "knocked-out gene" has led to compensatory changes in the developmental physiology of the animal with the result that the knockout phenotype ceases to be a reliable indicator of the function of the knocked out gene. This concern is particularly acute in the case of TRPV4 knockout animals because of the importance of TRPV4 in homeostatic mechanisms. Put simply, in the absence of the development of compensatory physiological changes, what we know of TRPV4 – not least in relation to its function in the cardiovascular system – would suggest that its efficient deletion throughout the entire animal would prove lethal to the animal.

It appears that neither muscular strength, exercise capacity, nor sensory inputs are affected in TRPV4 knockout mice. Likewise, absence of TRPV4 does not affect normal learning behaviour in the Barnes maze. Nor does it affect anxiety-like behaviour or locomotor activity in the light/dark transition test, the open field test, or the elevated plus maze test. However, animals lacking TRPV4 exhibited increased activity in the Porsolt forced swim test, which is interpreted as indicating decreased "depression-like behavior". Somewhat paradoxically, TRPV4 knockout animals also demonstrated decreased social behaviors in the tests used (354). The significance of such conclusions from studies on mice in relation to human affective disorders is not clear.

9. TRPV4 Function in Various Organ Systems

(i) Cardiovascular system

Understanding of the role of TRPV4 in mediating vascular tone and arterial blood pressure is facilitated by a brief recapitulation of the relevant structural and functional aspects of these vessels. The innermost layer of the artery comprises a single layer of endothelial cells. This is surrounded by vascular smooth muscle which is, in turn, surrounded by a coat of connective tissue. Successive branching of muscular arteries results in terminal arterioles which comprise endothelium surrounded by a single cell layer of smooth muscle. These supply capillaries which are endothelial tubes devoid of smooth muscle but with an abluminal layer of contractile pericytes. Small muscular arteries and arterioles comprise the principal resistance vessels in the circulation. This resistance, and both blood pressure and tissue blood

flow, are regulated through contraction or relaxation of vascular smooth muscle in response to a diverse range of signals, including factors derived from the endothelium and perivascular adipose tissue, and the activation of ion channels **(84, 86, 308, 363)**. TRPV4 plays a role in controlling vascular resistance through a variety of actions on different tissues.

(a) Endothelial cell function

The endothelium dynamically regulates vascular smooth muscle tone by means of the vasoactive substances which it secretes **(407)**. Moreover, endothelial cells express several enzymes and transport mechanisms which act on circulating hormones, including angiotensin converting enzyme (ACE), which catalyses the conversion of angiotensin 1 to angiotensin 2, an important vasoconstrictor **(51)**. Impaired endothelial function (endothelial dysfunction) in resistance vessels is now known to be a feature of a range of major cardiovascascular diseases, including hypertension and the complications of diabetes **(143)**. The endothelium also regulates thrombus formation and breakdown and plays a crucial role in the generation of new blood vessels (angiogenesis), as well as controlling capillary permeability and tissue fluid exchange **(87, 323, 444)**. In each case, increases in endothelial $[Ca^{2+}]_i$ are key to the generation of the downstream signals **(288, 386)**, which include NO, prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF) and endothelium-derived epoxyeicosatrienoic acids (EETs), substances which regulate vascular tone and thrombogenesis **(67, 104, 123, 275, 471)**. There is now considerable evidence that endothelial TRPV4 channels play an important role in the control of endothelial $[Ca^{2+}]_i$ and vascular function **(289)**. Some of the key features of endothelial and TRPV4 dependent vasodilatation are illustrated in **Figure 7.**

TRPV4 is expressed in endothelium *in situ* and in endothelial cells freshly isolated from small mesenteric arteries (**(263)**; **Figure 8**). Flow, an important vasodilatory stimulus, increases $[Ca^{2+}]_i$ in endothelial cells, with the $[Ca^{2+}]_i$ response varying with different flow conditions **(111, 165, 171)**. Shear stress, when applied to cultured human endothelial cells, causes TRPV4 channels localised near the nucleus to translocate to the cell membrane **(235)**. Shear-induced increases in endothelial $[Ca^{2+}]_i$ are inhibited by ruthenium red, which blocks TRPV4, and by knockdown of TRPV4 using short interfering RNA. TRPV4-mediated Ca^{2+} entry is also observed when HEK-293 cells transfected with TRPV4 are stimulated by flow. Endothelial Ca^{2+} is increased by the application of GSK1016790A, a

selective TRPV4 agonist which also induces marked dilation of small mesenteric arteries from normal mice, but not those from mice lacking TRPV4. Likewise, luminal flow in normal animals results in dilation which is endothelial-dependent, involving the release of both NO and EDHF, but these responses are markedly reduced in TRPV4 null mice **(263)**. Shear stress evokes oscillatory increases in $[Ca^{2+}]_i$ in endothelial cells and activates Ca^{2+}-sensitive K^+ channels, resulting in hyperpolarizing transient outward currents. These responses are dependent on Ca^{2+} influx which, in turn, induces Ca^{2+}-release from ryanodine-sensitive intracellular stores **(171)**. Activation of TRPV4 with 4α-PDD dilates carotid arteries and *arteria gracilis* in an endothelium-dependent manner while TRPV4 antagonists inhibit shear-stress-dependent vasodilation of those vessels **(203)**. Flow-induced vasodilation in mouse carotid arteries is reduced in TRPV4 knockout animals but only when NOS and cyclooxygenase are blocked. Under control conditions, i.e., in the absence of any blocker, the response to flow does not differ significantly between normal and TRPV4 knockout mice **(235)**.

Remarkably, the hyperpolarization and dilation of blood vessels induced by acetylcholine is reduced by ~75% in mesenteric resistance arteries in $TRPV4^{-/-}$ mice. In normal mice, 11,12-EET activates a TRPV4-like current and hyperpolarises the membrane of vascular smooth muscle cells, resulting in the dilation of mesenteric arteries. When the endothelium is disrupted, 11,12-EET-induced hyperpolarization and vasodilatation is reduced by approximately half. A similar effect is seen when either endothelial (small and intermediate conductance), or smooth muscle (large conductance) Ca^{2+}-activated K^+ channels are blocked. 11,12-EET is devoid of effect on membrane potential, diameter, or ionic currents in the same arteries taken from TRPV4 knockout mice **(104)**. Other studies have demonstrated a role for PKCα in the acetylcholine-dependent activation of TRPV4 and consequent vasodilatation **(1)**. However, the hypotensive action of acetylcholine is not blocked by a TRPV4 antagonist *in vivo* **(310)**. **In vitro experiments using aortic rings suggest that mild hypothermia activates TRPV4 resulting in acetylcholine production by the endothelium, which, in turn, activates muscarinic receptors on nearby cells to contribute to endothelium-dependent relaxation *in vitro.* However, this TRPV4-dependence was seen in rings from spontaneously hypertensive rats but not in normotensive controls (481).** There is also evidence that TRPV4 may co-assemble with other TRP subunits in endothelial cells to form heteromeric channels sensitive to flow and other dilatory stimuli, presumably increasing the complexity and diversity of signalling **(98)**.

Targeted signalling may also result from co-localization of relevant elements. **The micro-compartments constituted by the caveolae of human microvascular endothelial cells are enriched with TRPV4 and small-conductance K^+_{Ca} 2.3 channels. Loss of Cav-1 (which can act as a scaffolding protein within caveolar membranes) is associated with a reduction in EDHF-mediated vasodilation after exposure to shear stress and acetylcholine, suggesting this is functionally significant (139).**

When a fluorescent Ca^{2+}-indicator protein is expressed in vascular endothelium and the region adjacent to the cell membrane imaged using total internal reflection fluorescence (TIRF) microscopy, the ingress of Ca^{2+} through individual TRPV4 channels in the vascular endothelium of resistance arteries appears as local Ca^{2+} signals (or "sparklets"). Detailed analysis of these signals suggests that TRPV4 channels exist within four-channel clusters and exhibit co-operative gating. The amplification effect of this co-operativity, when coupled with the large TRPV4 single channel Ca^{2+}-conductance and the high Ca^{2+}-sensitivity of the small and intermediate conductance endothelial K^+-channels activated by the TRPV4 Ca^{2+}-signal, results in a high level of functional signalling sensitivity. Activation of as few as three to eight TRPV4 channels per cell can maximally dilate resistance arteries **(374)**. Mathematical modelling of vasodilatory signalling by TRPV4 involving these "sparklets" indicates that the opening of one individual TRPV4 channel causes a stochastic localised increase in Ca^{2+} in an area comprising some few μm^2 near to the ion channel. Further predictions include the occurrence of micromolar increases in Ca^{2+} extending over the channel's open period which are capable of activating low-affinity KCa^{2+} channels in the endothelium. Simulations of burst and co-operative gating forces involving a cluster of four TRPV4 channels predict quantal ("step") increases in Ca^{2+} comparable to the Ca^{2+} sparklets experimentally observed. It is these localised Ca^{2+} events which result in endothelium-derived hyperpolarization, the size of which is dependent on the frequency of these events **(312)**.

Reductions in intraluminal pressure, in the absence of flow, can also increase endothelial TRPV4 activity, resulting in dilation through activation of intermediate conductance K^+ channels in the endothelium. This may contribute to the autoregulation of blood flow in the face of changing perfusion pressures, promoting increased flow by decreasing vascular resistance at low pressures, and so augmenting the myogenic response **(22).** Endothelial TRPV4 channels appear to be involved in inhibiting α_1-adrenergic vasoconstriction in feed

arteries of skeletal muscle in response to increases in temperature, and so may help explain heat-induced sympatholysis **(137)**.

Myoendothelial projections (MEPs) are areas in which the membrane of endothelial cells crosses the internal elastic lamina of arteries to make close contact with smooth muscle cells. Gap junctions formed by connexins provide low resistance intercellular pathways for both electrical and chemical signalling at these sites. There is growing evidence that MEPs function as specialised signalling microdomains allowing endothelium and smooth muscle to communicate in both forward and reverse directions **(107, 406).** These regions express high levels of both PKC and the PKC-anchoring protein AKAP150 **(375)**. When muscarinic acetylcholine receptors on endothelial cells of mouse arteries are stimulated, they only activate TRPV4 channels that are localised at these MEPs. There is an increased Ca^{2+} influx at these projections as a result of co-operative opening of clustered TRPV4 channels. The same level of co-operative activation of TRPV4 channels is not found in other areas and is diminished when intracellular Ca^{2+} is chelated or when AKAP150 is knocked out. Muscarinic receptor stimulation fails to activate TRPV4 channels, co-operation between TRPV4 channels at myoendothelial projections is diminished and vasodilation in response to muscarinic receptor stimulation is reduced when localisation of AKAP150 at MEPs is disrupted in a mouse model of angiotensin II-induced hypertension **(375)**. However, TRPV4 channel signalling does not appear to be exclusive to MEPs. Channel expression in the endothelium of rat uterine radial arteries is not colocated with the holes in the internal elastic lamina. Both TRPV4 expression and the contribution of EDHF to vasodilatory responses in uterine arteries are increased in pregnant rats, again supporting a functional role for the channel in endothelium-dependent dilation **(346)**.

TRPV4 channels have been implicated in the regulation of endothelial structure and help maintain the normal orientation of endothelial cells, with their long axis parallel with the direction of flow. Cyclically stretching capillary endothelial cells activates TRPV4. This stimulates downstream phosphatidylinositol 3-kinase and induces binding of additional *B*1 integrin receptors to promote remodelling of the cytoskeleton and reorientation of the endothelial cells. Strain-induced capillary cell reorientation is suppressed by integrin inhibition and TRPV4 knockdown using siRNAs **(400)**. The initiation of Ca^{2+} influx through TRPV4 channels when force is applied to *B*1 integrins is astonishingly fast, occurring within 4 msec. Deformation of the lipid bilayer or sub-membranous cortical cytoskleton alone is not adequate to activate the TRPV4 channels. It has been suggested that binding of CD98 to the

distal integrin tail in the cytoplasm, thereby increasing the strength of focal adhesions, is necessary to ensure the presence of sufficient mechanical connectivity within the focal adhesion to allow TRPV4 channels to respond to the stresses applied to the extracellular domain of adjacent cell surface integrins **(262)**. TRPV4 may also play a role in regulating the endothelial barrier between blood and the surrounding tissue. TRPV4 agonists have been shown to increase lung endothelial permeability, a response that is absent in TRPV4$^{-/-}$ mice. This suggests that TRPV4 activation may be one mechanism underlying acute lung injury, possibly by activating matrix metalloproteases **(15, 415, 446)**. Pulmonary oedema secondary to high pulmonary venous pressure, typically caused by left ventricular failure, is also associated with TRPV4 activation in pulmonary capillaries in rodent models **(185, 458)**. This suggests TRPV4 channels are a promising target in the treatment and prevention of lung damage and pulmonary oedema **(192, 401, 415)**.

There is emerging evidence that TRPV4 plays an important role in vascular repair and remodelling. The channel has been implicated in the formation of collateral circulation around sites of arterial blockade. Activation of endothelial TRPV4 by flow shear-stress results in an increase in channel expression and encourages active proliferation of vascular cells that ultimately result in collateral vessel growth **(341, 342, 408)**. Circulating human endothelial progenitor cells – whose function is to repair damaged blood vessels and substitute for injured endothelial cells – express functional TRPV4 before their incorporation into vessels **(97)**. There is also evidence supporting a role for TRPV4 in tumor angiogenesis, with increased expression in tumor endothelial cells from human breast cancers. Knockdown of the TRPV4 channel prevents arachidonic acid-induced endothelial cell migration, one of the processes required for angiogenesis **(122).**

A wide range of cardiovascular diseases, including hypertension, diabetic vasculopathies and atherosclerosis are associated with endothelial dysfunction, with diminished endothelially-mediated vasodilatation and reduced antithrombogenic activity **(40, 345, 405)**. A number of observations have implicated loss of TRPV4 activity in the pathogenesis of endothelial dysfunction. The dilatory responses to both TRPV4 agonists and acetylcholine are reduced in small resistance arteries from an angiotensin II mouse model of hypertension and this is associated with a failure of TRPV4 activation in the MEPs **(375)**. Endothelial expression of TRPV4 and downstream Ca^{2+}-sensitive K^{+}-channels (K(Ca)2.3) are reduced in mesenteric arteries and retinal arterioles of streptozotocin-induced diabetic rats, while culturing microvascular endothelium in a hyperglycaemic environment downregulates both

structural and functional TRPV4 expression **(245, 274)**. TRPV4- mediated vasodilatation in cerebral arteries is also diminished in a number of different animal models of cerebrovascular pathology related to Alzheimer's disease **(469)**. It has been speculated that TRPV4 may play a role in renal ischaemia/reperfusion injury and that upregulation of endothelial TRPV4 contributes to the protective effect of hypoxic pre-conditioning **(193, 330)**. It seems reasonable to conclude that TRPV4 is emerging as a potentially important contributor to vascular pathology.

(b) Vascular smooth muscle cell function

TRPV4 is also expressed in systemic and pulmonary arterial smooth muscle where it may promote relaxation and vasodilation or contraction and vasoconstriction **(455)**. TRPV4 in cerebrovascular smooth muscle forms a Ca^{2+} signaling complex with ryanodine receptors and BK_{Ca} channels. Endothelial-derived 11,12-EET dilates cerebral arteries, an effect that is abolished by suppression of TRPV4 in intact cerebral arteries. EET activates TRPV4 in smooth muscle, leading to Ca^{2+}-induced Ca^{2+} release and activation of BK_{Ca} channels. The resulting hyperpolarisation of the smooth muscle causes vasodilatation by reducing L-type Ca^{2+}-channel activation **(103)**. Other studies support the conclusion that TRPV4, TRPC1 and BK_{Ca} form an EET sensitive, vasodilatory molecular complex within systemic vascular smooth muscle **(248, 471)**.

Activation of TRPV4 in the smooth muscle of pulmonary arteries, on the other hand, appears to induce a pro-constrictor effect **(Figure 9).** Hypoxia evokes constriction in distal pulmonary arteries (hypoxia-induced pulmonary vasoconstriction) and prolonged hypoxia can result in irreversible pulmonary hypertension. The TRPV4 agonist 4α-PDD elicits a contractile response in pulmonary arteries and this is potentiated under hypoxic conditions **(80)**. Consistent with this functional response, TRPV4 expression in pulmonary artery smooth muscle is increased in response to hypoxia, with a parallel increase in the myogenic responses to transmural pressure in endothelium-denuded vessels, leading to the suggestion that this may contribute to pulmonary hypertension **(446)**. On the other hand, short-term culturing of rat pulmonary artery smooth muscle under hypoxic conditions has been found to increase TRPV4 Ca^{2+}-signalling and cell migration without increasing ion channel expression. This is associated with hypoxia- induced nuclear translocation of the NFATc4, part of a transcription complex regulating gene expression, suggesting that TRPV4 plays an

upstream role in hypoxic signalling in these cells, and may be involved in the pathogenesis of pulmonary hypertension **(314)**. TRPV4 appears to be necessary for hypoxic pulmonary vasoconstriction (HPV), since inhibition or genetic knockdown of TRPV4 reduces the increase in pulmonary arterial pressure due to hypoxia. Blockade of TRPV4 in pulmonary arterial smooth muscle cells prevents Ca^{2+} influx and phosphorylation of myosin light chain (MLC) **(141)**. MLC phosphorylation is important in several cell functions, including vascular smooth muscle contraction **(195)**. Constrictor responses to serotonin are also reduced both following pharmacological blockade of TRPV4 and in TRPV4-/- mice, as is the enhancement in serotonin responses seen following chronic hypoxia **(447)**. Others have suggested that TRPV4 activation by serotonin may promote smooth muscle cell proliferation in pulmonary arterial smooth muscle **(101)**.

There is also evidence that TRPV4 can promote constrictor as well as dilator responses to certain stimuli in systemic vascular smooth muscle. The A kinase anchor protein AKAP150 is a scaffolding protein which is involved in local control of L-type Ca^{2+} channels found in arterial smooth muscle. In arterial myocytes, AKAP150-targeted protein kinase C_{alpha} signalling controls "stuttering persistent" Ca^{2+} entry through these channels, as well as increased vascular tone and blood pressure under normal conditions. It also mediates angiotensin II-dependent hypertension **(285)**, and increased persistent Ca^{2+} sparklet activity is found in arterial myocytes in hypertension **(287).** TRPV4 channels are gathered into *puncta* of differing sizes along the sarcolemma of arterial myocytes. Although basal TRPV4 sparklet activity is low, Ca^{2+} entry during elementary TRPV4 sparklets is 100-fold greater than that during L-type CaV1.2 channel sparklets. The activity of TRPV4 sparklets in specific regions of the cells can be increased by administering the TRPV4 channel agonist GSK-1016790A or angiotensin II (AngII). However, PKC and AKAP150 are required for AngII-induced increases in TRPV4 sparklet activity. The existence of dynamic subcellular signalling domains – comprising AKAP150, PKC, and TRPV4 – that control Ca^{2+} influx into arterial myocytes has also been proposed on the basis of evidence that initiation of AngII signalling increases the proximity of AKAP150 and TRPV4 *puncta* in arterial myocytes, while local stimulation of diacylglycerol and PKC signalling results in TRPV4-mediated Ca^{2+} influx **(265)**. TRPV4 mediates vasoconstriction in the aorta of mouse in conjunction with an agonist at the Tx receptor which is generated by cyclo-oxygenases and depends upon signalling by MAPK as well as Src kinase. The vasoconstriction induced is increased by means of EGF receptor activation and the involvement of PKC leading to the activation of

the G-protein coupled receptors for angiotensin or proteinases (AT_1 and PAR_1/ PAR_2, respectively) **(335)**.

(c) Astrocyte function and neurovascular coupling

Neurovascular coupling refers to mechanisms linking neuronal activity to vasomotor tone in the brain and retina, and plays an important role in the local control of cerebral blood flow to meet changing metabolic needs **(120)**. Astrocytes, which contribute to the coupling process, have been shown to express functional TRPV4 channels **(35)**. As in endothelial cells, signalling to vascular smooth muscle is triggered by a rise in astrocyte $[Ca^{2+}]_i$, and TRPV4 channels in the astrocytic end feet contribute to this rise **(102)**. TRPV4 also performs a regulatory function in relation to the permeability of the blood cerebrospinal fluid barrier and transepithelial protein transport. In pigs, this ion channel is broadly expressed on the apical membrane of choroid plexus epithelial cells which are found in the ventricles of the brain and comprise the blood cerebrospinal fluid barrier. Application of the TRPV4 agonist, GSK-1016790A, causes a major ingress of Ca^{2+} accompanied by serine/threonine phosphorylation. It also reduces filamentous actin and results in disintegration of cell junctions of the blood-cerebrospinal fluid barrier within 10-20 minutes. When normal TRPV4 activity is inhibited using the antagonist, HC-067047, there is a reduction in the basolateral-to-apical transport of α-2-macroglobulin (A2M) **(284)**.

(d) Heart

Human coronary arterioles express endothelial TRPV4 which are functionally related to both flow-mediated dilation and the production of reactive oxygen species **(47)**. There is limited evidence to date for a direct role of these channels in cardiac myocyte function. Exposure to hypotonic solutions increases cardiac contractility and modulates Na^+-currents. These effects are inhibited by ruthenium red, a non-selective inhibitor of TRPV channels **(173, 223).** TRPV4 expression in cultured cardiac myocytes is mainly nuclear with translocation out of the nucleus in response to hypotonic stimulation **(479)**. TRPV4 (and TRPC1) are expressed in sensory nerve endings in the atrial endocardium where they are coincident with the nerve ending vesicle marker synaptophysin **(352).** Perhaps the most significant observation to date is that TRPV4 is functionally active in cardiac fibroblasts and is involved in the differentiation of cardiac fibroblasts to form myofibroblasts in response to

tumour growth factor-β (TGF-β) signalling **(2, 159)**. This offers the prospect that myocardial repair and remodelling may be modified through selective TRPV4 activation.

TRPV4 is found in the endocardium while heart valve development is occurring and is essential for normal heart valve development. Oscillatory flow activates TRPV4 which regulates the Ca^{2+} response of endocardial cells and expression of the klf2a promoter, an atheroprotective transcription factor which also responds to flow. Similar findings have been made with respect to TRPP2 which is known to associate with TRPV4. The absence of either TRPV4 or TRPP2 is associated with valve defects, suggesting they play an important role in valve formation **(162)**.

(e) *In vivo* effects and BP control

Consistent with its ability to dilate blood vessels and reduce peripheral resistance, TRPV4 exerts a hypotensive effect when activated. Indeed, generalised systemic activation of TRPV4 can be catastrophic. When the TRPV4 agonist GSK-1016790A is given intravenously in mouse, rat or dog¸ it has no direct effect on cardiac contractility or rate but causes a dose-dependent fall in blood pressure, indicating a fall in resistance. This is followed by major circulatory collapse associated with failure of the permeability barrier constituted by the pulmonary microvasculature **(441)**. **On the other hand, the plant alkaloid, berberine, which is an inhibitor of TRPV4, induces vascular relaxation and lowers blood pressure in both mice with steroid induced hypertension and aged apolipoprotein E knockout mice. These effects were reversed by overexpression of TRPV4 (429). Knockout mice which lack the K^+_{Ca} 3.1 channel -- an intermediate conductance Ca^{2+}/CaM-regulated K^+ channel – avoid the fatal pulmonary circulatory collapse otherwise induced by pharmacological activation of TRPV4 in normal animals. Hence, inhibition of these Ca^{2+} -activated K^+ channels may prove protective against dangerously high endothelial Ca^{2+} signalling induced by excessive activation of TRPV4 (427).** Although basal blood pressure is similar in wild type and TRPV4 knockout animals, the transient hypertension produced by inhibition of NOS is greater in mice which lack TRPV4, suggesting that TRPV4 channel activity opposes hypertensive stimuli *in vivo* **(104).** GSK-1016790A dilates blood vessels and reduces arterial pressure in both pulmonary and systemic vascular beds in rat. When NOS is inhibited, however, the same TRPV4 agonist doses cause a more marked fall in systemic pressure but evoke pulmonary vasoconstrictor responses that are reduced by

isradipine, an L-type Ca^{2+} channel antagonist. This suggests that TRPV4 activation has different actions in pulmonary and systemic vascular beds **(311)**. Intravenous injection of acetylcholine decreases blood pressure by more than twice as much in normal mice as it does in TRPV4 knockout mice whereas pharmacological blockade of TRPV4 does not inhibit the hypotensive action of acetylcholine in rats **(310, 467)**. The physiological significance of TRPV4 to cholinergic control of pressure control, therefore, remains unclear.

The effects of TRPV4 in the intact animal will reflect the net effect not just on endothelium and vascular smooth muscle but also any TRPV4-mediated changes in nervous or hormonal control of the cardiovascular system. TRPV4 activation reduces blood pressure in Wistar rats but this effect is exaggerated in animals fed on a high salt diet. TRPV4 expression in the sensory nerves of the DRG and mesenteric arteries is increased in these animals, as is release of the vasodilatory transmitters calcitonin gene-related peptide (CGRP) and substance P. This is consistent with a compensatory mechanism in which TRPV4 activation opposes the hypertensive effects of salt intake **(126)**. The hypotensive response to intravenous administration of the TRPV4 agonist 4α-PDD is reduced in animals lacking capsaicin-sensitive sensory nerves and in animals to which a CGRP antagonist has been administered, as well as in animals which have been given a combined dose of blockers of small, intermediate, and large conductance Ca^{2+}-activated K^+ channels. Agonist activation of TRPV4 increases plasma levels of the vasodilator transmitter CGRP in normal rats and this may contribute to the hypotensive effect. Interestingly, TRPV4 is found co-localised with CGRP in vascular sensory neurons as well as with Ca^{2+}-activated K^+ channels in the endothelium of mesenteric resistance arteries **(128)**.

A potential antihypertensive role of TRPV4 has also been tested using Dahl salt-sensitive and salt-resistant rats, which are differentiated by their relative sensitivity to the hypertensive effects of a high salt diet. Activation of TRPV4 reduces mean arterial pressure in male Dahl salt-resistant rats on a high sodium diet but has considerably less hypotensive effect in Dahl salt-sensitive rats on a high sodium diet. A high salt diet increases TRPV4 expression in Dahl salt-resistant rats but decreases it in salt-sensitive animals, and blockade of TRPV4 increases blood pressure in Dahl salt-resistant rats on a high sodium diet only. This suggests that differential control of TRPV4 expression and function may explain, at least in part, the differences in salt-sensitivity which have been found (**127)**. In other circumstances, however, TRPV4 appears to have a hypertensive role, e.g., the increase in blood pressure after drinking water is effected by activation of the sympathetic nervous system, with

TRPV4 being an essential component of that response **(251)**. When given l-NAME in drinking water for seven days, TRPV4 null mice exhibited a slightly greater increase in mean arterial pressure when compared to wild type controls. However, in mice given subcutaneous infusion of Ang II for 14 days, similar increases were found in mean arterial blood pressure in both groups of animals. Thus, TRPV4 appears to have a minor role in regulating blood pressure in the hypertension induced by l-NAME; and does not appear to function in regulating hypertension induced by Ang II **(300)**.

(ii) Respiratory system

Expression of TRPV4 in the epithelium of the foetal lung increases during the course of gestation. TRPV4 has been implicated in the mechanism by which mechanical stress engages an inflammatory response and resulting injury in the foetal lung. TRPV4 controls the release of the cytokine IL-6 through pathways which involve p39 or ERK. TRPV4 is also involved in the regulation of stretch-occasioned differentiation of epithelial cells in the foetus **(286)**. Functional TRPV4 is expressed in human airway smooth muscle cells **(184, 249)**, and is also present in a human bronchial epithelial cell line **(118)**. Extracellular Ca^{2+} influx following agonist-induced activation of TRPV4 in epithelial cells results in the activation of Ca^{2+}-dependent K^+ channels, which are important in the regulatory volume decrease (RVD) after hypotonic stress. The Ca^{2+} signal in this context is capable of being generated away from the location of Ca^{2+}-dependent K^+ channels at the plasma membrane of epithelial cells and is not the result of release of intracellular Ca^{2+} *via* ryanodine receptors **(118)**. In airway epithelial cells of cystic fibrosis sufferers, however, swelling of these cells fails to initiate Ca^{2+} ingress through TRPV4 channels thereby contributing to failure of RVD **(20)**. The TRPV4 ion channel is of particular functional importance in ciliated epithelia **(16)**. The channels are mainly expressed on the ciliary membrane of ciliated tracheal epithelium. Ciliary beat frequency is heightened by increases in $[Ca^{2+}]_i$, governing the rate at which mucus is cleared in the airways. Ca^{2+}-signalling in response to 4α-PDD and moderate temperatures is reduced in ciliated epithelial cells of the trachea of TRPV4(-/-) mice, although this does not prevent autoregulation of ciliary beat frequency in the presence of highly viscous solutions. The facilitation of Ca^{2+} entry by TRPV4 is also implicated in increases in ciliary beat frequency induced by ATP, suggesting a role in epithelial receptor operated Ca^{2+}-entry **(236)**. Activation of TRPV4 in the smooth muscle of human airway evokes contractions which are inhibited by a TRPV4 antagonist but, also, by a 5-lipoxygenase blocker and by two cysteinyl

leukotriene 1 receptor antagonists which have been shown to be structurally distinct. It has been suggested that activation of TRPV4 causes airway constriction that is reliant upon the production of cysteinyl leukotrienes **(249)**.

TRPV4 is implicated in several pulmonary disease states in addition to cystic fibrosis **(140)**. The endothelial cells of rat main pulmonary artery express TRPV4 and its activation by a pharmacological agonist results in strong concentration-related relaxation of the endothelium-intact pulmonary artery. This TRPV4-mediated endothelium-dependent vasodilation is contributed to by NO and EDHF **(384)**. Agonist-induced activation of TRPV4 and ingress of Ca^{2+} is attenuated in rat pulmonary microvascular endothelial cells after pre-treatment with an inhibitor of myosin light chain kinase, while surface TRPV4 expression is reduced by pre-treatment with the same inhibitor **(313)**. TRPV4-mediated Ca^{2+} entry in septal endothelium increases lung endothelial permeability **(416, 446)**. TRPV4 is expressed in the alveolar septal wall in human, rat, and mouse, and there is evidence that its agonist-induced activation can result in its participation in acute lung injury. In isolated rat lung, agonist-induced activation of TRPV4 increases endothelial permeability in the lung in a dose- and Ca^{2+}-dependent manner in rat and mouse, resulting in disruption of the endothelial and epithelial layers of the alveolar septal wall with blebs or breaks **(15)**. In accord, fast ingress of Ca^{2+} *via* TRPV4 is a major factor in determining the extent of the increase in vascular permeability in lungs after high peak inflation pressure ventilation **(155)**. Filtration coefficients measured in isolated perfused lungs after high peak ventilation pressure show no significant increase in mice lacking the TRPV4 receptor, but exhibit a more than two-fold increase in lungs taken from normal animals. This may depend on the expression by alveolar macrophages of TRPV4 **(154)**. Inhalation of nanoparticles releasing a TRPV4 inhibitor serves to prevent ventilator damage in mice for several days **(192)**. Increased microvascular pressure in lung, as is seen in left ventricular failure, heightens endothelial $[Ca^{2+}]_i$ by TRPV4 activation which results in increased lung vascular permeability. The endothelial $[Ca^{2+}]i$ activates myosin light-chain kinase and at the same time stimulates NO synthesis to transiently increase the filtration coefficient. In mice lacking TRPV4, increases in endothelial $[Ca^{2+}]_i$ induced by pressure are largely blocked, as are the synthesis of NO and the lung wet/dry ratio. Endothelial NO formation provides a negative feedback mechanism which limits the increase in permeability by a cGMP-dependent inhibition of TRPV4 activity **(458)**. Matrix metalloproteinases (MMPs) may also play a role in TRPV4 mediated lung pathophysiology, regardless of the underlying cause. These enzymes are involved in the

proteolytic processing of extracellular matrix structural proteins. Activation of TRPV4 and the resulting Ca^{2+} influx thereby occasioned leads to the activation of MMP2 and MMP9 which damage cell-to-cell or cell-matrix adhesion. This increases the permeability of the septal barrier and thereby contributes to lung injury **(415)**.

TRPV4 also appears to participate in the pathology of pulmonary hypertension of varying causes. TRPV4 is up-regulated in pulmonary arteries of rats within one day of hypoxia exposure; and activation of TRPV4 results in an increase in the Ca^{2+} response in hypoxic pulmonary arterial smooth muscle cells. Hypoxia-induced pulmonary hypertension, right ventricular hypertrophy and vascular remodelling are reduced in TRPV4 null mice **(454)**. There is an increase in TRPV4 expression in the pulmonary vasculature in human heart failure patients. Moreover, blockade of TRPV4 inhibits heightened vascular permeability and consequential pulmonary oedema associated with heightened pulmonary venous pressure **(401)**. TRPV4 is important in mediating the enhanced pulmonary vasoreactivity to serotonin which is seen in chronic hypoxic pulmonary hypertension **(447)**. Both TRPV1 and TRPV4 are expressed in rat intrapulmonary arteries and their agonist-induced activation results in increased $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells. Stimulation of these ion channels induces pulmonary arterial smooth muscle cell migratory responses which are associated with cytoskeletal reorganisation which may result in the increased pulmonary vascular resistance characteristic of pulmonary hypertension **(257)**. The TRPV4 and RyR2 expression found in pulmonary arterial smooth muscle cells is increased in the same cells of chronically hypoxic rats. Blockade of the ryanodine receptor significantly reduces the increase in $[Ca^{2+}]_i$ and the arterial contraction which results from agonist activation of TRPV4 **(80)**. NOS appears to regulate the responses to activation of TRPV4 differently in pulmonary vascular beds and systemic vascular beds, respectively. L-NAME increases the hypertensive effect of agonist activation of TRPV4 on the systemic circulation but converts agonist activation of TRPV4 in the pulmonary circulation from inducing an hypotensive effect to an hypertensive effect **(311)**. The shear stress-mediated rise in $[Ca^{2+}]_i$ and the protein expression level of TRPV4 (and TRPM7) channels are greater in the pulmonary arterial smooth muscle cells of sufferers of idiopathic pulmonary arterial hypertension than in healthy individuals. Blockade of TRPV4 by ruthenium red inhibits shear stress-induced rise in $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells in patients with both normal blood pressure and in those with idiopathic pulmonary arterial hypertension. 4α- PDD-induced activation of TRPV4 results in a greater increase in $[Ca^{2+}]_i$

in pulmonary arterial smooth muscle cells of patients with such hypertension than in non-hypertensive patients. Knockdown of TRPV4 (and TRPM7) by siRNA attenuates the shear stress-mediated $[Ca^{2+}]_i$ increases in both myocyte types **(372)**.

TRPV4 has been implicated in the development of idiopathic pulmonary fibrosis, which is a fibrotic lung disease resulting from the development of myofibroblasts. Myofibroblast generation, in turn, is dependent on mechanical signalling and activation of TGF-β1. Lung fibroblasts obtained from patients with idiopathic pulmonary fibrosis exhibit increased TRPV4 activity, while mice which lack TRPV4 do not suffer from lung fibrosis. When TRPV4 is inhibited either by genetic ablation or pharmacologically, then differentiation of myofibroblasts fails to occur – a phenomenon which is reversed by the reintroduction of TRPV4 **(328)**.

These channels also seem to play a role in lung injury following exposure to chemical irritants. Post-exposure treatment with a TRPV4 inhibitor suppresses pulmonary inflammation in response to acid or chlorine gas by diminishing neutrophils, macrophages and associated chemokines and cytokines, while improving tissue pathology (**Figure 10)**. These findings are confirmed using knockout mice which lack the TRPV4 receptor. TRPV4 inhibitors likewise produce similar anti-inflammatory effects in chlorine-exposed mice where they also improve blood oxygen saturation. Increased concentrations of N-acylamides, a class of endogenous TRP channel agonists, are found in both models of lung injury **(25)**. Studies using the TRPV4 inhibitors, GSK-2220691 or GSK-2337429A, in TRPV4 deficient mice, confirm this ion channel as an important contributor to inflammation of the lung resulting from direct chemical injury. These inhibitors have also been shown to exert vasculoprotective effects when administered in the aftermath of exposure to chlorine gas, preventing leakage from blood vessels while increasing the oxygenation of the blood **(278). In the context of acid-induced acute lung injury, the absence, or inhibition, of TRPV4 reduces the platelet activating factor-induced increases in neutrophil $[Ca^{2+}]_i$ and neutrophil responses (including ROS formation, neutrophil adhesion and chemotaxis) resulting from pro-inflammatory stimuli are inhibited (459).**

TRPV4 has also been implicated in obstructive airways disease. Increased expression of TRPV1 and TRPV4 is found in tissue samples taken from the lungs of patients suffering from chronic obstructive pulmonary disease (COPD). Blockade of the TRPV1, TRPV4 and pannexin-1 channels reduces the increase in ATP released from primary airway bronchial

epithelial cells as a result of cigarette-smoking **(29)**. In severe asthma, airway smooth muscle cells are caused to proliferate, contributing to the alteration and permanent obstruction of airways. TRPV4 agonists increase the proliferation of primary airway smooth muscle cells. Ca^{2+} sparklets resulting from opening of single TRPV4 channels generate targetted Ca^{2+}-signalling microdomains in these cells. TRPV4 channels are co-expressed with calcineurin in primary airway smooth muscle cells. Activation of calcineurin results in the dephosphorylation of nuclear factor of activated T cells (NFAT) transcription factors cytosolic (c) to permit nuclear translocation and the activation of synthetic transcriptional pathways. Activation of TRPV4, which results in airway smooth muscle cell proliferation is accompanied by calcineurin-dependent nuclear translocation of the NFATc3 isoform. Ca^{2+} microdomains created by TRPV4 Ca^{2+} sparklets have been proposed to activate calcineurin to induce nuclear translocation of NFAT and airway smooth muscle cell proliferation **(475)**.

(iii) Urinary system

(a) Kidney

The early study by Liedtke and Friedman (2003) reported that mice lacking the TRPV4 channel exhibit impaired osmotic regulation. They drink less water, and become hyperosmolar when compared to wild-type animals under control conditions and when exposed to hyperosmolar challenges, with reduced levels of antidiuretic hormone despite increased osmolarity. TRPV4 null animals demonstrated smaller reductions in drinking and experienced systemic hypotonicity when subjected to continuous subcutaneous infusion of the antidiuretic hormone analogue, dDAVP at dosages that had no effect on control animals. **(230)**. However, these effects were relatively small in magnitude and were only revealed in animals housed individually without food to remove group and feeding influences. A more recent study found no evidence for a role of TRPV1 and TRPV4 in thirst responses to hypertonic stimuli in single knockout animals for either gene or double knockouts for both **(199).** The reason for this discrepancy remains unclear as very similar protocols were used in both cases.

TRPV4 has an extensive distribution in the renal vasculature **(68)**. Moreover, TRPV4 is expressed at the cell membranes of cultured M-1 collecting duct cells and at the luminal membrane of mouse kidney collecting duct cells **(445)**. TRPV4 is found in cells of the

human collecting duct but such expression is reduced by approximately half at 48 hours after exposure to high glucose, and this can only be accounted for in part by the resulting increase in osmolarity occasioned by the glucose **(168).** A PKA-dependent cascade promotes TRPV4 trafficking and translocation to the apical membrane and increases channel activity in the distal nephron **(253)**. Activation of TRPV4 by luminal flow increases intracellular calcium and causes NO to be produced in the thick ascending limb in rat **(49, 50)**. TRPV4 and TRPP2 assemble to form a 23-pS divalent cation-permeable non-selective ion channel at the apical membrane of renal principal cells of the collecting duct. The TRPV4/TRPP2 complex exhibits a different biophysical amd pharmacological profile to that of either of its constituent channels. Epidermal growth factor (EGF) stimulates the TRPV4/TRPP2 channel through EGF receptor (EGFR) tyrosine kinase-dependent signalling. In addition, EGF increases cell proliferation in cells which do not possess normal cilia and this is dependent upon a TRPV4/TRPP2 channel mediated increase in $[Ca^{2+}]_i$ **(474)**.

A synergistic interaction has been proposed to occur in mouse cortical collecting duct M-1 cells between TRPV4, the small conductance SK3 (K(Ca)2.3) channel and the large conductance BK channel (K(Ca)1.1). When the cells are depolarised by selective inhibition of the BK channel (by iberiotoxin) or by selective inhibition of the SK3 channel (by apamin), TRPV4-mediated Ca^{2+} influx is found to depend on the activation of both K^+ channels **(188)**. Selective activation of TRPV4 in cortical collecting duct cells in mouse gates both SK3 channels and the BK channels and this results in hyperpolarization of the cell membrane **(38)**. TRPV4 is important in causing the sustained elevation of $[Ca^{2+}]_i$ during purinergic stimulation *via* P2Y2 receptors in aldosterone-sensitive distal nephron cells, as evidenced by the fact that an ATP-induced Ca^{2+} plateau is dramatically reduced in TRPV4 null mice **(254)**.

TRPV4 activity is reduced in freshly isolated collecting duct cysts in rat models of polycystic kidney disease. This is associated with reduction in basal $[Ca^{2+}]_i$, and a failure of flow-mediated $[Ca^{2+}]_i$ signalling, as well as an alteration in the location of TRPV4 within the cell. The renal symptoms of autosomal recessive polycystic kidney disease (ARPKD) are reduced by agonist-induced augmentation of TRPV4 activity systemically over time. Selective activation of TRPV4 reinstates mechanosensitive Ca^{2+} signalling and restores TRPV4 functioning and localisation within the cell. Thus, pharmacologically-induced increase in the activity of TRPV4 in the longer term slowly reinstates sensitivity to mechanical stimuli in cyst cells and substantially reduces renal ARPKD progression. There appears to be a connection between impairment of TRPV4-dependent mechanosensitivity in

collecting duct cells and cystogenesis which suggests that there may be a therapeutic value to manipulating TRPV4 activity pharmacologically in order to slow the progression of ARPKD **(463)**. An associated finding is that biliary cholangiocytes from rats with polycystic kidney disease exhibit a substantial over-expression of TRPV4, as do the livers of (ARPKD) disease patients, although the significance of this remains unclear **(146).**

(b) Bladder

TRPV4 is found in mouse and rat urothelium (which is the epithelium covering the surface of the urinary bladder) and in the vascular endothelium of the bladder. TRPV4 is important for normal bladder voiding, and mice lacking this receptor are incontinent and show, on cystometry, a lower frequency of voiding contractions as well as a higher frequency of non-voiding contractions **(136)**. Functional TRPV4 is found in the urothelium lining the renal pelvis, ureters, urinary bladder, and urethra **(39)**. TRPV4 in the urothelium of the mouse bladder is mainly found at the basal plasma membrane domains of the basal urothelial cells (**452)**, and is prominently localised to the abluminal surfaces of the umbrella cells **(462).** TRPV4 is also expressed in the suburothelium and suburothelial nerve plexus of the urinary bladder and in small- and medium-sized lumbosacral (L1, L2, L6-S1) DRG cells from mice; and such expression is increased by over-exposure to NGF **(138)**. TRPV4 is also found in detrusor muscle in rat and may be involved in detrusor over-activity associated with bladder outlet obstruction in rat **(74)**. TRPV4 is not co-localised with TRPV1 in sensory neurons serving the bladder, but each of these channels is present in a different population of bladder afferents. Bladder hyperactivity resulting from lipopolysaccharide-induced cystitis remains unaffected by separate low-dose treatment with an antagonist of TRPV4 or TRPV1 (176.7 ng/kg RN-1734 and 143.9 ng/kg SB-366791, respectively). However, co-administration of both antagonists at the same low dose completely reverses bladder hyperactivity caused by lipopolysaccharide-induced cystitis and partially reverses cystitis-induced bladder pain behaviour **(64)**. TRPV4 is expressed in urinary bladder smooth muscle where it is important in bladder contraction. Agonist-induced activation of TRPV4 with GSK-1016790A causes the contraction of mouse bladders *in vitro* as well as bladder hyperactivity **(402)**. TRPV4 plays a role in bladder dysfunction resulting from stress. When rats are subjected to repeated but varied stresses over a period of a week there is a resulting increase in the level of TRPV4 found in urothelium. On the other hand, inhibition of TRPV4

receptors reduces the extent of bladder dysfunction resulting from such stress **(267)**. The TRPV4 agonist, GSK-1016790A (0.1 μM), has been shown to increase the amplitude of spontaneous contractions in bladder strips of rat and may have a role in the treatment of underactive bladder disease **(461)**. TRPV4-KO mice exhibit a markedly higher voiding frequency and a lesser volume of urine when voiding than wild-type animals. However, the TRPV4-KO mice have a greater urine output than normal mice. TRPV4-KO animals generate bladder contractions which do not occasion voiding in advance of each successful voiding, while normal mice do not generate similar bladder contractions **(460)**.

Agonist-induced activation of TRPV4 in rat urinary bladder urothelial cells evokes Ca^{2+} ingress, promotes ATP release, and increases the ATP released by hypo-osmolarity. In awake rats during continuous infusion cystometrograms, intravesically given 4α-PDD (10-100 microM) substantially increases micturition pressure **(39)**. Application of stretch, *via* a cell-stretch system, to cultured mouse primary urothelial cells results in $[Ca^{2+}]i$ increase and ATP release, effects which are reduced in cells lacking TRPV4 **(272)**. Activation of TRPV4 by intravesical application of GSK-1016790A participates in the micturition reflex in rat by activation of the C-fibers of primary bladder afferents which are mechanosensitive but non-responsive to capsaicin **(4)**. Use of the TRPV4 antagonist, HC-067047, can increase functional bladder capacity and reduce micturition frequency in normal mice and rats with cystitis **((110)**; **Figure 11**).

(iii) Musculoskeletal system

(a) Bone and cartilage

There is increasing evidence that TRPV4 plays an essential role in regulating various musculoskeletal tissues **(150).** TRPV4 is found in both osteoblasts (which synthesise bone) and in osteoclasts (which resorb bone tissue) **(269)**. Activation of TRPV4 in osteoclasts increases both their number and their resorption activity and this results in bone loss **(258)**, while absence of TRPV4 reduces bone resorption thereby increasing bone mass **(259)**. Where patients are confined to bed, they may develop so-called “unloading-induced osteoporosis” where the absence of load on bones, which are normally accustomed to bear it, results in a reduction in bone mass. Removing the mechanical stress of normal load-bearing from the hind-limbs of naïve mice results in osteopenia, while this phenomenon fails to occur in animals which lack TRPV4. Absence of TRPV4 prevents the diminution in the

rates of mineral apposition and of bone formation induced by loss of normal load-bearing. It also prevents the rise in the number of osteoclasts in primary trabecular bone and the shortening of its longitudinal length which is otherwise caused by such "unloading" **(269)**. TRPV4 controls the end-stage differentiation of osteoclasts and their activity **(259)** and higher levels of TRPV4 expression are found on differentiation of osteoblasts in culture **(390)**. Activation of TRPV4 reciprocally controls Ca^{2+}/CaM signalling, which involves TRPV4 associating with non-muscle myosin IIa **(258)**. TRPV4 appears to mediate the Ca^{2+} signalling in osteoblasts which is induced by flow **(390)**.

TRPV4 may be a male-specific determinant of bone strength. In male mice lacking the TRPV4 receptor there is a 20 per cent increase in mass in cortical and trabecular bones, but intracortical porosity is heightened and there is a reduction in bone matrix mineralisation. The end result is that the presence or absence of TRPV4 is irrelevant to measures of maximum load, stiffness and work to failure of the femoral bone. However, the bone material exhibits a lower resistance to stress and less elasticity in TRPV4 null mice. When compared with normal female mice, TRPV4 null female mice do not show any of these variations in skeletal measurements **(411)**.

Ca^{2+} signalling mediated by TRPV4 appears to be essential for healthy joints and the maintenance of a normal skeletal structure. Male mice lacking TRPV4 experience major osteoarthritic abnormalities which are age-related and dependent on male sex. Again, such mice exhibit heightened sub-chondral bone volume and heightened calcified meniscal volume **(78)**.

Chondrocytes are cells found in cartilage where they produce and maintain the cartilaginous matrix. Functional TRPV4 are found in articular chondrocytes and mediate the responses of these cells to hypo-osmotic stress **(318)**. Mice lacking TRPV4 fail to exhibit the Ca^{2+} responses to hypo-osmotic stress exhibited by chondrocytes of normal animals **(78)**. TRPV4 in chondrocytes embedded in agarose gel are important in determining how those chondrocytes respond to dynamic compression **(181, 304)**. TRPV4 is involved in the regulation of Sox9, which is a transcription factor required for differentiation in chondrocytes and for chondrocyte-specific gene expression **(281)**. Activation of TRPV4 has been employed in tissue engineering to improve the tensile properties of self-assembled articular cartilage constructs **(106)**. The collagen-binding receptor integrin $\alpha 1\beta 1$ is important in mediating the response of chondrocytes to hypo-osmotic stress by alterations in $[Ca^{2+}]_i$.

TRPV4 is expressed on normal and integrin α1-null chondrocytes but is only capable of being activated on the normal chondrocytes **(181)**. The phosphorylation of ERK1/2 is important in determining the level of TRPV4 expressed in chondrocytes subjected to hypo-osmotic stress. Inhibition of ERK1/2 using the specific blocker (PD-98059) inhibits an increase in the number of TRPV4 channels in response to hypo-osmotic stress. It also reduces the level of TRPV4 below the expression level of controls in iso-osmotic conditions **(161)**. The expression of TRPV4 by chondrocytes of the temporomandibular joint in rat is inhibited by an over-expression of microRNA-203 (miR-203), while the expression of NO in the same cells is increased, apparently by this micro-RNA targeting the *Trpv4* gene **(172)**.

Type VI collagen is not required for Ca^{2+} signalling *via* TRPV4 in mouse, but loss of type VI collagen changes the mechanical features of the pericellular matrix and this age-dependently increases cell swelling and the signalling by TRPV4 which is induced by hypo-osmotic pressure **(465)**. There is evidence that the mechanical qualities of neocartilage constructs may be improved at cellular and tissue levels in tissue engineering by the use of hyperosmolarity and the TRPV4 agonist, 4α-PDD. In combination, 4α-PDD interacts with hyperosmolarity to promote its impact on collagen content as well as tensile strength **(221)**.

(b) Muscle

TRPV4 is functionally expressed in mouse skeletal muscle **(169, 324)**. Skeletal muscle contraction is essentially regulated by release of Ca^{2+} and its removal into the sarcoplasmic reticulum; and TRPV4 activation in skeletal muscle governs resting Ca^{2+} influx and muscle fatigue **(324)**. Absence of TRPV4 results in heightened metabolic capacity in mouse skeletal muscle and resistance to weight-gain due to a high-fat diet. Loss of activity of TRPV4 appears to result in compensatory increases in TRPC3 and TRPC6 production in skeletal muscle, as well as heightened calcineurin activity, in TRPV4 null mice when compared to normal animals **(209)**. Lengthening contraction, which can cause soreness and pain on movement after the passage of time, causes mechanical hyperalgesia in wild type mice within 6 to 24 hours, but fails to do so in mice which lack either TRPV1 or TRPV4 **(307)**.

(v) Skin

TRPV4 is found at the site of formation of *adherens* junctions in skin keratinocytes where it functions with *beta*-catenin which links these cell-to-cell junctions with the actin cytoskeleton to enhance their development and ensure the formation of a tight barrier between skin keratinocytes. In the absence of TRPV4 in the area of cell-to-cell junction formation, abnormal cell-to-cell junction formation occurs and structures develop which result in increased intercellular permeability *in vitro* **(370)**. TRPV4 mediates the entry of Ca^{2+} from the extracellular space at normal skin temperature to cause Rho activation which encourages the organisation of actin fibres and the formation of junctions to heighten the integrity of the barrier formed by these cell-to-cell junctions **(90, 371).** TRPV4 is essential for the development and maintenance of the barrier constituted by skin epidermal keratinocytes using tight cell-to-cell junctions, which avoids excess dehydration of human skin. Activation of TRPV4 by normal skin temperature, or by an agonist, enables the ingress of Ca^{2+} from extracellular spaces which encourages the development of cell-to-cell junctions **(197)**. TRPV4 activation reinforces the epidermal tight junction barrier between human epidermal keratinocytes in culture **(5)**. UVB radiation increases the expression and activation of epidermal TRPV4 resulting in damage to the skin and an increase in endothelin-1 which mediate the pain of sun-burn **(276)**. **Expression of TRPV4 is enhanced in two sub-types of the inflammatory facial skin disease known as rosacea, namely, papulopustular rosacea and phymatous rosacea (Sulk et al., 2012).**

Functional TRPV4 receptors are found in human sebocytes **(305)**. Moreover, cannabidiol has been found to induce lipostatic and anti-proliferative effects from its activation of TRPV4 expressed by these cells **(305)**. When cannibidiol activates TRPV4 expressed by sebocytes, it disrupts the prolipogenic ERK1/2 MAPK pathway and down-regulates nuclear receptor interacting protein 1 (NRIP1). These effects alter glucose and lipid metabolism resulting in inhibition of sebocyte lipogenesis. The anti-inflammatory actions of cannabidiol are related to A2a adenosine-dependent up-regulation of tribbles homolog 3 (TRIB3) and inhibition of NF-kB signalling. These findings suggest that cannabidiol may prove an effective treatment for acne vulgaris **(305).**

(vi) Head and neck

(a) Submandibular gland

TRPV4 is expressed in the mouse submandibular gland where it is found in the acinar cells. Activation TRPV4 in the submandibular gland results in Ca^{2+} ingress and salivation (**472)**.

(b) Eye

Functional TRPV4 expression is found in human endothelial cells of the cornea **(266).** It is also found localised at the surface of the cell membrane and in the cytosol of human epithelial cells of the cornea and the activation of these ion channels plays a role in relation to RVD in these cells **(309)**. TRPV4 is expressed in neurons of trigeminal ganglion nerve endings in the inner walls of the anterior chamber of the eye in rat **(264).** TRPV4 is found in retinal ganglion cells and at the optic nerve head in mouse, as well as in Muller glial cells **(333)**. TRPV4 is expressed in the apical microvilli of a small group of cells of cultured human foetal retinal pigment epithelium **(478)**.

TRPV4 mediates the effects of the inositol polyphosphate 5-phosphatase, OCRL, in the cells of the trabecular meshwork in the eye which are involved in the control of intraocular pressure, and a pathological OCRL allele is believed to inhibit Ca^{2+} signalling through TRPV4 **(241)**. TRPV4 is expressed in the epithelium of the porcine lens and functions in the reaction of the lens to hypo-osmotic stress. ATP is released from the intact lens when it is subjected to an hypo-osmotic solution, with a more than threefold increase in its concentration being found in the bathing solution. This release is blocked by HC-067047 and RN-1734, both of which are antagonists of TRPV4. Consistent with the involvement of TRPV4 in ATP release, GSK-1016790A, an agonist of TRPV4, effects release of ATP when the lens is exposed to isosmotic solution. A hypo-osmotic solution also activates Src family kinase which also increases Na^{+}-K^{+}-ATPase activity in the lens epithelium – effects which are also blocked by TRPV4 antagonists. The ATP does not exit the cell *via* TRPV4 in response to hypo-osmotic shock. The ATP molecule is too large for this to occur. Instead, TRPV4 causes hemichannel-mediated release of ATP **(347, 348, 349)**. "Hemichannels" describe the half of a gap junction channel (formed from connexin proteins) that is provided by one cell. Hemichannels are regarded as being functional when they are open in non-junctional membranes without pairing with a partner from an adjacent cell **(377)**. Surprisingly, Ca^{2+} ingress in response to hypo-osmotic activation of TRPV4 appears to occur *via* connexin hemichannels, presumably as a result of a TRPV4 activation-induced

conformational change occasioned in these hemichannels which facilitates Ca^{2+} influx through these channels. Interestingly, the resulting increase in $[Ca^{2+}]_i$ is kept within physiological limits even though these hemichannels have a large capacity to facilitate entry into the cell of Ca^{2+} ions **(255)**. Astrocytes of the optic nerve head in rat, when subjected to mechanical strain, release ATP *via* pannexin 1 channels. This is proposed to account for the high level of extracelluar ATP exhibited in the optic nerve head of glaucoma patients **(30)**. **TRPV4 appears to enable epithelial cells of the porcine lens to identify damage to the tightly-packed fibre mass which comprises most of the lens. It then promptly activates Src family kinases and increases Na^+, K^+-ATPase activity which allows the lens to improve its regulation of ion concentrations in the fibre mass. It has been suggested that the mechanosensitive TRPV4 may be activated as a result of swelling of the lens structure resulting from fibre damage (350).** TRPV4 channels are also functionally expressed in the endothelium of retinal microvessels. This expression is down-regulated in a rat model of type 1 diabetes and channel function is reduced in bovine endothelial cells exposed to high glucose in culture, suggesting that TRPV4 may be a useful molecular target in diabetic retinopathy **(274).** This pathogenic link remains to be established.

Melatonin is found in the eye where it is involved in the regulation of intraocular pressure. TRPV4 may be involved in regulating extracellular melatonin in the eye since activation of TRPV4 by its agonist GSK-1016790A has been found to produce an increase in the level of extracellular melatonin in cells of a cell line derived from human non-pigmented ciliary epithelial cells **(14)**.

(c) Ear

Hearing involves exceptionally sensitive mechanosensation which is dependent on specific ion channels which transduce vibrations induced by sound. In mammals, TRPML3, TRPV4, and TRPC3/TRPC6 have been identified as being involved in the functioning of the inner ear **(464)**. TRPV4 in the guinea pig and in mouse is expressed in the hair cells and supporting cells of the organ of Corti, in marginal cells of the stria vascularis, spiral ganglion cells, sensory cells, transitional cells, dark cells in the vestibular end organs, vestibular ganglion cells and epithelial cells of the endolymphatic sac **(178, 398)**. TRPV4 null mice have normal hearing at 8 weeks, but exhibit impairment of hearing by 24 weeks **(392)**. Hearing loss is a known accompaniment of TRPV4 mutation-induced neuropathy in some

instances and it has been suggested that TRPV4 may be essential for maintaining cochlear function in stress conditions, such as exposure to noise capable of causing acoustic injury **(306)**. TRPV4 ion channels are involved in aminoglycoside uptake and retention in the inner ear **(196, 220)**. Both TRPV1 and TRPV4 ion channels are involved in the trafficking of gentamicin in cochlear hair cells. Gentamicin results in damage to cochlear hair cells in the rodent in a manner which reflects duration of exposure to the drug and drug-dose. Increased vulnerability to gentamicin is found in hair cells located at the basal turn as compared to hair cells located at the apical turn **(220)**.

(d) Nose

TRPV4 is found expressed in the olfactory epithelium in mouse **(3, 282)**, but in the methimazole-treated mouse, the expression of TRPV4 is reduced and recovers only over time **(282)**. Activation of TRPV4 in human nasal epithelial cells results in an increase in ciliary beat frequency. However, challenge with a TRPV4 agonist, GSK-1016790A, can cause an increase in ciliary beat frequency followed by cell death **(6)**.

(e) Larynx

TRPV4 is expressed in laryngeal epithelial cells in both mouse and human **(152, 153)**.

(f) Mouth

Odontoblasts are found at the edge of the dental pulp. These cells are responsible for the process of dentine formation throughout life **(18)**. TRPV4 are found in minor collections of these cells within a general odontoblast population taken from adult rat **(210)**. When an odontoblast cell is exposed to mechanical stimulation, $[Ca^{2+}]_i$ increases as a result of the activation of TRPV1, TRPV2, TRPV4 and TRPA1 **(357)**.

(vii) Digestive system

(a) Oesophagus

Functional TRPV4 expression is found in keratinocytes of the mouse oesophagus, and agonist-induced activation of these ion channels increases the release of ATP from these keratinocytes **(268)**. TRPV4 is also expressed in human oesophageal tissue as well as in HET-1A, an epithelial cell line **(409)**.

(b) Gastrointestinal tract

DRG neurons innervating the colon express TRPV4 which, when activated by 4α-PDD, result in a visceral hypersensitivity which is dose-dependent **(58)**. The human colon expresses TRPV4. Mouse colon which has become inflamed expresses an increased level of TRPV4 compared with control non-inflamed tissue. When 4α-PDD is introduced within the colon in mouse, colitis is found after 3 to 6 hours, and in live animals an increase in the permeability of the colon is found 3 hours later. However, the induced inflammation clears within 24 hours **(82).** Pre-treatment of DRG neurons innervating the colon with either histamine or serotonin enhances the responses of neuronal TRPV4 to activation by 4α-PDD. The same treatments increase the expression of TRPV4 at the plasma membrane. Abolition of TRPV4 reduces the hypersensitivity resulting from administration of serotonin or histamine **(59)**. Inflammatory bowel disease in humans is accompanied by an increased level of TRPV4 expression in the gastrointestinal tract. Blocking TRPV4 in a mouse model of colitis reduces colitis and pain **(119)**. Cenac and colleagues have confirmed the importance of TRPV4 activation in mediating the visceral hypersensitivity found in irritable bowel disease (IBS). Higher levels of 5,6-EET (an agonist of TRPV4) are found in tissue taken from the colons of patients suffering from IBS. Supernatants of IBS biopsies cause visceral hypersensitivity in mice which is inhibited where TRPV4 has been eliminated in mice primary sensory neurons by siRNA knockdown. Polyunsaturated fatty acid metabolites whether derived from IBS biopsies or from the colons of mice suffering from visceral hypersensitivity activated primary sensory neurons of mice *in vitro* by activating TRPV4. These authors also found that TRPV4 is expressed in 35% of neurons in human DRG **(60)**.

Activation of TRPV4 reduces resistance across intestinal epithelium. The non-steroidal anti-inflammatory drug (NSAID), indomethacin, produces a similar effect resulting in increased permeability of the intestinal epithelium in part by occasioning the activation of TRPV4 by metabolites of arachidonic acid. Hence, TRPV4 is implicated in damage to the intestine resulting from the use of NSAIDs **(453)**.

(c) Pancreas

TRPV4 is found in cells of the murine pancreatic beta cell line MIN6 and in mouse pancreas. Abolition of TRPV4 avoids the increase in $[Ca^{2+}]_i$ otherwise induced by human amyloid polypeptide (hIAPP), and reduces both the endoplasmic reticulum stress response and level of cell death induced by hIAPP **(55)**. Agonist-induced activation of TRPV4 results in a swift increase in $[Ca^{2+}]_i$ and an increase in the insulin secretion induced by glucose in INS-1E pancreatic beta cells. When TRPV4 is inhibited, the same activators fail to increase $[Ca^{2+}]_i$ and insulin secretion in these cells **(368)**. More than two-thirds of the pancreatic stellate cells isolated from rats with pancreatitis caused by a diet of high-fat and alcohol have increased levels of TRPV4 and increased Ca^{2+} responses when exposed to TRPV4 activators **(470)**. **The TRPV4 antagonist, HC-067047, reduces the hypersensitivity experienced by rats suffering from chronic pancreatitis induced by an alcohol and high fat diet which results in the animals developing referred visceral pain-like behaviours. TRPV4 may therefore constitute an appropriate target for therapeutic intervention designed to relieve the pain of pancreatitis (468).**

(d) Liver

The epithelial cells of the bile duct are known as "cholangiocytes." These cells have primary cilia at the apical membrane which extend into the lumen of the ducts. Cholangiocyte cilia express TRPV4 which respond to luminal tonicity to secrete bicarbonate, the principal factor governing the formation of ductal bile, by a process which involves apical ATP release. Agonist stimulation of TRPV4 results in an increase in the flow of bile, in addition to the release of ATP and the secretion of bicarbonate **(145)**. TRPV4 expression is increased in the cholangiocytes of rats suffering from polycystic kidney disease. When TRPV4 are activated in these cells, the result is an increase of 30 per cent in $[Ca^{2+}]_i$, as well as inhibition of cell proliferation by some 25%-50% and a three-fold reduction in cyst growth in 3-dimensional culture. However, activation of TRPV4 *in vivo* results in a reduction in renal cystic area but not in liver cysts **(146)**. In donor liver of rat preserved for 12 hours before transplantation, the expression of TRPV4 in the bile ducts disappears completely after 8 weeks, a phenomenon which is not found where the donor liver is preserved for 1 hour only before transplantation (**240)**. TRPV4 expression is substantially increased in fibrotic liver tissue and proliferation of HSC-T6 cells caused by TGF-*B*1 is

reduced by blockade of TRPV4 **(373)**. Application of TGF-*B*1 results in greatly enchanced expression of TRPV4 in HSC-T6 cells. Transfection of these cells with small interfering RNA (si-TRPV4) to prevent the expression of TRPV4, results in the cells exhibiting an increase in apoptosis and inhibition of autophagy. On the other hand, when these cells are treated with the TRPV4 agonist, 4α-PDD, the result is reduction of apoptosis and enhanced autophagy. Autophagy is believed to be induced by TRPV4 by its regulation of Akt signalling **(466)**.

(e) Metabolism

Human preadipocytes express high levels of TRPV4 which participate in adipogenesis through the phosphorylation of p-Akt kinase. Silencing either of TRPV4, TRPV2 or TRPM7 individually results in reduced adipogenic differentiation of preadipocytes through reducing the availability of p-Akt kinase **(66)**. Whether TRPV4-deficient mice are protected from diet-induced obesity is unclear. Ye and colleagues (2012) found that mice lacking functional TRPV4 exhibit heightened thermogenesis in adipose tissues and do not suffer from obesity resulting from their diet. Moreover, these authors concluded that these animals are also saved from resistance to insulin and, interestingly, from inflammation of adipose tissue **(457)**. Kusuda and colleagues (2012) found that TRPV4-deficient mice demonstrate a heightened oxidative capacity in skeletal muscle and are protected against obesity resulting from a high-fat diet. These authors suggested that loss of TRPV4 results in compensatory increases in the production of TRPC3 and TRPC6, as well as an increase in calcineurin activity, which alters energy metabolism by means of heightened expression of genes which function in the oxidation of fuel in skeletal muscle. This is suggested to result in heightened expenditure of energy, as well as affording protection from obesity occasioned by a high-fat diet **(209)**. O'Conor and colleagues (2013) reported that, in mice which do not possess the TRPV4 ion channel, a high-fat diet results in an increase in the severity of knee osteoarthritis, as well as reducing their normal cage activity. It also increases their weight gain and results in increased adiposity. Bone marrow-derived stem cells and adipose-derived stem cells from TRPV4 deficient mice also exhibit a change in differentiation potential. Bone marrow-derived stem cells have reduced adipogenic and osteogenic differentiation potential when compared with similar cells from wild-type animals. Adipose-derived stem cells from mice lacking TRPV4 possess an increased adipogenic potential as well as a reduced chondrogenic potential. These authors point to significant differences between their study and that of

Kusuda and colleagues **(209),** including the age of the animals when the high-fat diet was commenced, the composition of the diet, and period during which it was fed to the animals **(303)**. Various factors associated with the development of obesity are involved in modulating the activity of TRPV4. Thus, hypo-osmolarity evokes a TRPV4-mediated, mainly inward, current, an increase in $[Ca^{2+}]_i$ and membrane depolarisation in bovine articular chondrocytes which is reduced by insulin, TNF-α, IL-1β, and, to a lesser extent, resistin and leptin **(338)**. Moreover, an association has been found between a TRPV4 gene variant and obesity in a study of human subjects in Taiwan. It has been suggested that this association should be considered in the context of the high expression of TRPV4 found in the hypothalamus which is known to be involved the regulation of body mass **(100)**.

Castellani and colleages (2014) have confirmed a role for TRPV4 in the development of inflammation in adipose tissue occasioned by inflammatory insult. This may be countered, in part, by the subject mice having previously undergone an exercise regime. Training mice on a treadmill over several weeks results in an increase in the indicators of mitochondrial content in skeletal muscle, as well as the development of PPAR-gamma coactivator 1 alpha in epididymal adipose tissue. The genes for IL-6, SOCS3 and TNF-α induced in epididymal adipose tissue by acute inflammation are reduced by ~50% in mice which are exercise-trained compared with animals which have not been so trained. In addition, the increases in plasma IL-6 found in the latter animals are reduced in the trained animals which also exhibit a reduced expression of TRPV4 **(56)**.

(viii) Nervous system

(a) Central nervous system

TRPV4 is widely distributed within the brain **(294, 412)**, being expressed in the endothelium of cerebral arteries where it regulates Ca^{2+} influx into endothelial cells under the influence of PLA_2 **(256)**. Increased expression of TRPV4 is found in cerebral collateral arteries after fluid shear stress stimulation, and agonist-induced gating of TRPV4 increases cerebral arteriogenesis **(342)**. Agonist-induced activation of TRPV4 governs $[Ca^{2+}]_i$ in the endothelial cells of human brain capillaries and contributes to the control of proliferation of those cells **(160)**. TRPV4 channels on endothelial cells effect the dilation in cerebral arteries caused by acetylcholine **(469)**. TRPV4 functions to regulate the activity of Na^+ channels in

hippocampal neurons. Intracerebroventricular injection of either of the TRPV4 agonists, GSK-1016790A or 4α-PDD, inhibits voltage-gated Na^+ current in hippocampal CA1 pyramidal neurons, shifting the voltage-dependent inactivation curve to hyperpolarization, with no effect on the voltage-dependent activation curve. Blocking PKA reduces GSK-1016790A-induced inhibition of this current while blocking PKC or p38 MAPK fails to alter the effect of GSK-1016790A. Sustained activation of TRPV4 results in increased expression of the α-subunit of voltage-gated Na^+ channels in the hippocampus. Hippocamapal Nav1.1, Nav1.2, and Nav1.6 levels are increased in TRPV4 agonist-injected mice, but Nav1.3 and Navβ1 levels are almost unaltered **(170)**. Intracerebroventricular injection of a hypotonic solution (270 mOsmol) of artificial cerebrospinal fluid (ACSF) reduces mean blood pressure compared to control mice or mice injected with 300 mOsmol of artificial cerebrospinal fluid. However, heart rate is not similarly affected. The reduction in blood pressure is eliminated by RN-1734, an antagonist of TRPV4, which indicates that activation of TRPV4 produces this depressor effect. This suggests that activation of TRPV4 can cause periventricular structures in the brain to effect a depressor response to such activation **(114)**.

TRPV4 functions in regulating the permeability of the blood-cerebrospinal fluid barrier (BCFSB) which comprises the choroid plexus epithelial cells of the brain ventricles. These cells control the passage of plasma proteins and immune cells into the central nervous system. In choroid plexus epithelial cells in swine, TRPV4 is found concentrated on the apical membrane. Treatment with the TRPV4-specific agonist, GSK-1016790A, occasions pronounced Ca^{2+} influx and instant serine/threonine protein phosphorylation. Within 10-20 minutes of this treatment, a pronounced diminution in the extent of filamentous actin is induced as well as disintegration of cell junctions. On the other hand, when basal TRPV4 activity is inhibited by a TRPV4-specific antagonist, there is a reduction in alpha-2-macroglobulin (A2M) transport in the basolateral-to-apical direction **(284)**.

Functional TRPV1 and TRPV4 are required to sustain normal excitability in murine mangocellular neurosecretory cells; and normal excitability becomes reduced in the absence of functional TRPV1 and TRPV4. Loss of functional TRPV4 does not affect heat-induced variations in the excitability of these cells, although loss of functional TRPV1 does do so **(383)**.

Functional TRPV4 is found in the cell membranes of cortical astrocytes in rat, particularly in membranes found between the brain and the fluid-filled spaces surrounding it

(35). TRPV4 ion channels are expressed in the endfeet of astrocytes where their Ca^{2+} signals are increased and transmitted as a result of Ca^{2+}-induced Ca^{2+} release from inositol 1,4,5-trisphosphate receptors. Ca^{2+} ingress effected *via* TRPV4 channels is a component of the Ca^{2+} response of astrocytic endfeet and increases vasodilation. TRPV4 is involved in regulating local blood flow within the brain to meet the requirements of neuronal activity **(102)**. It has been implicated in mediating the cell death of astrocytes caused by oxidative stress in cultured hippocampal slices **(23)**. TRPV4 channels expressed on astroycytes in the hippocampus can be gated by synthetic amyloid-beta 40 with resulting injury to neurons and astrocytes. The level of TRPV4 expression is increased and so also is the level of free Ca^{2+} found within astroycytes **(24)**. Seven days after cerebral hypoxic/ischaemic injury, the level of TRPV4 is substantially increased in astrocytes of the CA1 region of the rat hippocampus and this is accompanied by astrogliosis. The responses of astrocytes to 4α-PDD are also increased after hypoxic/ischaemic injury **(48)**. TRPV4 channels expressed in astrocytes appear to be important sensors of blood flow/pressure in parenchymal arterioles while purinergic signalling emanating from glia is proposed to contribute to alterations in the tone of parenchymal arterioles resulting from flow/pressure. However, TRPV4 does not appear to be expressed in the endothelium of parenchymal arterioles **(198)**. TRPV4 is expressed in cells of the immortalised neuroendocrine rat hypothalamic 4B cell line where its expression in the cell membrane can be enhanced by the effect of Ang-II on the Ang-1 receptor (AT1R) *via* a Src kinase pathway **(340).**

Alterations in TRPV4 expression in the central nervous system have been investigated using SOD1(G93A) mutant transgenic mice as an animal model of amyotrophic lateral sclerosis (ALS). An increased expression of TRPV4 is found in the cerebral cortex, hippocampal formation, thalamus, cerebellum and spinal cord of symptomatic SOD1(G93A) transgenic mice. Increased expression of TRPV4 can be identified in multiple brain regions of these animals and in the spinal cord, suggesting a role for TRPV4 in the pathology of this disease **(219)**.

Stroke generates various activators of TRPV4 and it has been suggested that inappropriate activation of this ion channel may have a damaging effect in the aftermath of a stroke. In a murine model of focal cerebral ischaemia, agonist-induced gating of TRPV4 increases NMDA-activated current in CA1 pyramidal neurons of the mouse hippocampus. The current thus evoked is affected by antagonists of TRPV4 and of NMDA receptors, respectively, suggesting that gating of TRPV4 increases activation of NMDA receptors **(224)**. TRPV4

effects the strengthening of synaptic transmission found in the hippocampus as a result hypotonic stimulation **(225)**.

Over-activation of microglia in the mouse brain by an injection of lipopolysaccharide into the mouse cerebral ventricle results in an increased release of TNF-α and increased expression of galectin-3. These increases in TNF-α and galectin-3 are reduced by concurrent agonist-induced activation of TRPV4 **(204)**. Infrasound – which is a form of low frequency noise capable of injuring neurons in rat – heightens the level of TRPV4 expressed in glial cells. Absence of functional TRPV4 in glial cells (astrocytes and microglia) in culture reduces the extent of IL-1β and TNF-α present and limits NF-kB nuclear translocation and apoptotic cell death in neurons. Hence, the view that TRPV4 expressed in glial cells may be involved in mediating neuronal injury resulting from infrasound **(353)**.

Co-expression of TRPV4 and TRPV1 is found in the cell bodies of a population of DRG neurons and in the central terminals of laminae I and II of the spinal dorsal horn in rat **(53)**. Anoctamin 1 is a Ca^{2+}-activated Cl^- channel which interacts both physically and functionally with TRPV4 in the apical membranes of epithelial cells of the choroid plexus to affect cell volume **(396)**.

Interestingly, it appears that TRPV4 expression throughout the brain and in the spinal cord of rats is increased with old age **(218)**. However, the significance of these age-related increases in the expression of TRPV4 in the central nervous system has yet to be elucidated.

TRPV4 is expressed in approximately one-third of the astrocytes which are found in the brain. Agonist-activation of these TRPV4-expressing astrocytes results in that activation spreading to adjacent astrocytes *via* gap junctions as well as by the release of ATP from the TRPV4-expressing astrocytes. In the aftermath of such activation, glutamate is released from both astrocytes which express TRPV4 and astrocytes which do not. This glutamate functions as an excitatory gliotransmitter which augments synaptic transmission *via* mGluR, type 1 **(356)**.

(b) Peripheral nervous system

TRPV4 is found in peripheral sensory neurons. TRPV4 protein is conveyed axonally to neuronal terminals at the periphery. TRPV4 mediates nociceptive responses to hypotonic

stimuli **(12)**. TRPV4 is found in DRG neurons **(388, 389)** and, in rat, it is also found co-expressed with TRPV1 in the cell bodies of a population of DRG neurons **(53)**. TRPV4 is generously expressed in trigeminal ganglion sensory neurons. It makes a crucial contribution to pain in mice caused by inflammation of the temporomandibular joint **(72)**. TRPV4 is also found in dural afferents in rat and gating of these ion channels by agonist or other activating stimulus causes allodynia of the animal's face and hind-paw **(439)**. Primary afferent neurons expressing TRPV4 innervate the colon; and PAR_2 activation results in mechanical hyperalgesia and excitation of sensory nerves of the colon only in the presence of functional TRPV4 **(44, 367)**. TRPV4 is also expressed in the *cauda equina* **(388)**, as well as in inner hair cells, outer hair cells, and spiral ganglion neurons of the mouse cochlea **(351, 398)**. TRPV4 is also found on sympathetic and parasympathetic nerve fibres, such as those innervating the arrector pili smooth muscle in skin, sweat glands, intestine, and blood vessels **(88)**. It is found in the perikarya, axons and dendrites of retinal ganglion cells and at the optic nerve head in mouse, as well as in Muller glial cells **(333)**. Sensory neurons innervating the blood vessels of the liver are capable of detecting hypo-osmotic variations in the osmolality of the blood. These neurons have their perikarya in the thoracic DRG and can accurately translate progressive alterations in osmolality by a mechanism which is dependent on TRPV4. In the absence of TRPV4, these neurons lose their capacity to generate inward osmosensitive currents and there is a complete loss of the capacity to activate peripheral osmoreceptors **(214)**.

TRPV4 is found in sensory and spinal motor neurons not only in the adult, but also in early development. Activation of TRPV4 promotes neurotrophic factor-derived neuritogenesis (i.e., formation of neurites) in developing peripheral neurons, while the loss of such activation has the contrary effect. NGF or cAMP treatment increases the level of expression of PLA_2 and TRPV4, and a PLA_2 –TRPV4 pathway has been proposed as a regulator of neurotrophic factor-derived neuritogenesis in developing peripheral neurons **(182)**.

(ix) Immune system

TRPV4 channels are expressed in T cells where their activation results in Ca^{2+} ingress into those cells. TRPV4 expression can be enhanced in T cells in the course of

concanavalin A-driven mitogenic and anti-CD3/CD28 stimulated TCR activation of T cells. It has been proposed that antagonism of TRPV4 may have a role to play in the regulation of mitogenic and T cell receptor mediated T cell activation as well as the resulting production of cytokines **(252).**

(x) TRPV4-evoked ATP release

Whereas ATP functions within the cell as an essential component of cell metabolism, extracellular ATP interacts in an autocrine or paracrine fashion with P2X receptors which are ATP-gated non-selective cation channels on the surface of cells which affect a range of physiological events **(65)**. Heat, mechanical stimulation and hypo-osmotic stress can cause intracellular ATP to escape in a TRPV4-dependent manner from within to outside the cell in several tissue types. Thus, the cilia of cholangiocytes express TRPV4 which, when activated by the TRPV4 agonist, 4α-PDD, causes the flow of bile to increase, ATP to be released and bicarbonate to be secreted **(145)**. Activation of TRPV4 in the oesophageal keratinocytes of mice results in Ca^{2+} ingress and the egress of ATP to outside the cell by exocytosis **(268)**. Reduced osmolality activates TRPV4 to cause the release of ATP from the thick ascending limb of the renal medulla **(364)**. Stretch-induced activation of TRPV4 in cultured primary urothelial cells results in Ca^{2+} influx and the secretion of ATP **(272)**. ATP secretion from urothelial cells into the bladder evoked by distension or LPS is contributed to by pannexin channels and this release affects voiding function **(31)**. TRPV4 activation also contributes to hypotonicity-induced release from airway epithelial cells of ATP *via* pannexin 1 channels **(344)**. The TRPV4 found in epithelium of the porcine lens is involved in mediating the response of the lens to hypo-osmotic conditions. In a hypo-osmotic solution, the lens releases ATP in a manner which is dependent on activation of TRPV4 and results in a greater than three-fold increase in the concentration of ATP in the solution. The ATP molecules exit the cell *via* hemichannels as a result of TRPV4 activation, being too large to exit *via* TRPV4. **(347, 348, 349)**. When astrocytes of the rat optic nerve head experience mechanical stress, ATP is released through pannexin-1 channels. This may explain why glaucoma patients are found to have increased extracellular ATP in the optic nerve head **(30)**. However, TRPV4 may not be invariably involved in the secretion of extracellular ATP. Airway smooth muscle cells are involved in the secretion of ATP in the airways in which ATP functions as an extracellular mediator. ATP is released when mechanical stretch is applied to cultured primary human airway smooth muscle cells. However, although cyclic stretch-induced

release of ATP is significantly reduced by inhibitors of Ca^{2+}- dependent vesicular exocytosis, this does not result from the application of a hemichannel blocker or from blockade of TRPV4 **(394)**. Activation of TRPV4 and TRPA1 in human odontoblast-like cells can stimulate the release of ATP **(105).**

Cigarette smoking causes primary airway epithelial cells of the bronchus to release increased levels of extracellular ATP in a dose-dependent manner. The extent of this release is reduced by blocking pannexin-1, TRPV1 and TRPV4 channels. In addition, mRNA expression of TRPV1 and TRPV4 is found to be enhanced in samples of tissues taken from patients suffering from COPD **(29)**.

(xi) TRPV4 and barrier function

TRPV4 functions in linking cell-to-cell junctions in skin keratinocytes with the actin cytoskeleton to ensure the development of a tight barrier between these keratinocytes **(5, 197, 370, 371)** (discussed at *Skin, supra*). Activation of TRPV4 results in reduction in the level of filamentous actin and disintegration of cell junctions between the choroid plexus epithelial cells of the brain ventricles which constitute the blood-cerebrospinal fluid barrier **(284)**.

10. Role of TRPV4 in Acquired Diseases

(i) Pain

TRPV4 plays an important role in mediating certain pain sensations due to inflammation or neuropathy.

(i) Inflammation-induced mechanical hyperalgesia

The inflammatory response typically results in local “mechanical hyperalgesia” in the area affected by the inflammation **(440)**. “Mechanical hyperalgesia” refers to a noxious sensation experienced after contact or pressure is applied to the inflamed area. TRPV4 is essential for the occurrence of such inflammation-induced mechanical hyperalgesia.

Injecting a mix of inflammatory mediators (inflammatory soup) fails to induce any significant mechanical or osmotic hyperalgesia in mice or rats which lack functional TRPV4 ion channels, but normal animals respond to such injections with an increase in pain-related behaviour **(8, 12, 70)**. TRPV4 is also involved in mediating inflammation-evoked pain in temporomandibular joint (TMJ) disorder in mice. When carrageenan is introduced by intra-articular injection into the rat TMJ, the levels of both TRPV4 and PAR_2 are increased. Administration of TRPV4 activators results in a dose-related increase in the extravasation of plasma and the extent of mechanical allodynia **(89)**. Injection into the TMJ of complete Freund's adjuvant results in inflammation in which the TMJ innervating trigeminal sensory neurons in wild-type mice exhibit an increase in both TRPV4 and phosphorylated ERK. Both of these effects are absent in mice which lack TRPV4 **(72)**. TRPV4 also has a role in eliciting the nocifensive behaviour which results from injection of formalin into the mouse whisker-pad. In experiments with isolated cells, formalin appears to activate TRPV4 directly to induce Ca^{2+} influx, and both TRPA1 and TRPV4 contribute to mediate trigeminal pain induced by formalin **(71)**. Cathepsin S is another product of the inflammatory response which can activate PAR_2 which then activates TRPV4. Its injection in mouse results in inflammation and hyperalgesia which is reduced by deleting either PAR_2 or TRPV4 or by inhibiting adenylyl cyclase **(476)**. Tyrosine kinases have been implicated in the activation of TRPV4 by PAR_2 **(144)**. Activation of PAR_2 creates a signal, probably *via* lipid mediators derived from arachidonic acid, which induces the activation of TRPV4 **(Figure 12)**. This functional coupling between PAR_2 and TRPV4 is necessary to maintain a sustained inflammatory response. PAR_2 activation of TRPV4 in this process is dependent on tyrosine phosphorylation which depends on an essential tyrosine residue,TRPV4-Tyr-110 **(322)**. Neutorophil elastase is another substance released at sites of inflammation as part of the inflammatory reponse. Neutrophil elastase activates PAR_2 which in turn causes Gαs-dependent activation of adenylyl cyclase and PKA which activates TRPV4 to occasion neurogenic inflammation and pain by sensitising nociceptors. Thus, neutophil elastase activates PAR_2 to sensitise TRPV4 currents generated in Xenopus laevis oocytes; and when elastase is given to mice by intraplantar injection it occasions oedema and mechanical hyperalgesia *via* activation of PAR_2 and TRPV4 **(477).**

TRPV4 is commonly found with TRPC1 and TRPC6 in DRG. Deletion of TRPV4 or of TRPC1 and TRPC6 eliminates the effect of inflammation in inducing hyperalgesia to mechanical or hypotonic stimulation without altering the normal threshold for mechanical

nociception. TRPC1 and TRPC6 are believed to function with TRPV4 to result in mechanical hyperalgesia and the sensitisation of primary sensory neurons **(7)**. Interleukin-17 (IL-17) increases the expression of TRPV4 in mouse DRG neurons and may thereby promote the development of mechanical hyperalgesia (**343)**.

TRPV4 in primary sensory neurons can readily translate increases or decreases in osmolarity into pain sensation. Thus, injection beneath the skin of the hindpaw in rats of 2% (607 mOsm) or 10% (3,250 mOsm) saline solution results in dose-related pain-induced behaviour, namely, flinching. In the presence of PGE(2), the flinching caused by 2% saline is increased by a multiple of 7; but the flinching caused by 10% saline remains unaffected by the presence of PGE(2). Deletion of TRPV4 reduces the flinching caused by 2% saline by 46% but has no effect on the extent of the flinching caused by 10% saline. Likewise, only the pain-related behaviour resulting from 2% saline is reduced in TRPV4 knockout mice. Hence, the suggestion that TRPV4 mediates pain sensation resulting from variations in osmolarity **(11)**. The use siRNA targeting TRPV4 has been shown to inhibit TRPV4 expression and function in DRG neurons in culture leading to the suggestion that it may have a role in the treatment of TRPV4-induced disease **(233)**.

TRPV4 is found abundantly in the sensory neurons innervating the colon. In mice, it is found co-localised with CGRP in a subpopulation of these sensory neurons **(44)** and also with PAR_2 **(367)**. TRPV4 contributes to the visceral pain response mediated by mechanical stimulation of colonic serosal and mesenteric sensory neurons **(44)**. The presence of TRPV4 is necessary for the excitation of colonic primary sensory neurons and the development of the mechanical hyperalgesia which is mediated by activation of PAR_2 in mouse **(367)**. Activtion of TRPV4 by its agonist, 4α–PDD, generates a cationic current and Ca^{2+} ingress in colonic primary sensory neurons in mouse to produce a dose-related visceral hypersensitivity **(58)**. Moroever, the visceral hypersensitivity induced by this agonist activation of TRPV4 in these primary afferents is aggravated by the sensitisation of TRPV4 effected by the presence serotonin or histamine **(59)**. Functional TRPV4 are found in intestinal epithelial cells of mice. Moreover, agonist-induced activation of these receptors occasions the development of colitis in mice within 3 to 6 hours **(82)**. Patients suffering from inflammatory bowel disease show higher levels of TRPV4 mRNA expression. In an animal model of this disease, blockade of this receptor relieves colitis and reduces the pain occasioned by inflammation of the intestine **(119)**.

TRPV4 and TRPA1 are found in DRG neurons serving the pancreas. These ion channels contribute to the pain of pancreatitis in mice, while TRPA1 also mediates pancreatic inflammation **(61)**.

The endogenous lipid molecule, Resolvin D1 (RvD1), reduces inflammation and the activation TRPV4 (as well as the activation of TRPV3 and TRPA1) at nanomolar concentrations. It may also function as an analgesic to relieve inflammatory pain **(28)**. In a mouse model of inflammatory pain, electroacupuncture at the ST36 acupoint reverses both mechanical and thermal hyperalgesia. Moreover, TRPV4 and TRPV1 protein levels in DRG neurons (which are increased at four days after injection of carrageenan or complete Freund's adjuvant) are reduced by such treatment while the threshold, rise-time and fall-time of action potentials in DRG neurons are indicative of a reduction in neuronal excitation and are consistent with an antinociceptive effect of treatment with electroacupuncture **(69)**.

(ii) Peripheral neuropathy-induced mechanical hyperalgesia or allodynia

TRPV4 contributes to mechanical hyperalgesia in several types of painful peripheral neuropathies, including that associated with taxol, paclitaxel, or vincristine chemotherapy, alcoholism, diabetes and human immunodeficiency virus/acquired immune deficiency syndrome therapy **(9, 10, 73)**. Alpha2- β1 integrin and Src tyrosine kinase are involved with TRPV4 in mediating such mechanical hyperalgesia and, indeed, a direct interaction occurs in sensory neurons between these several contributors to mechanical hyperalgesia **(9)**.

The mechanical allodynia resulting from paclitaxel-induced neuropathy in mouse is dependent on the activation of both TRPA1 and TRPV4. Mechanical allodynia caused by paclitaxel is diminished in part by the antagonist of TRPA1, HC-030031, and also by the TRPV4 antagonist, HC-067047. Moreover, it is wholly abolished by the combined administration of both of these antagonists **(260)**. Paclitaxel administration causes neuronal degeneration in DRG, as well as an increase in TRPV4 expression. Hyperalgesia resulting from paclitaxel-induced neuropathy is attenuated by administration of goshajinkigan which appears to achieve this effect by preventing neuronal degeneration and inhibiting increases in expression of TRPV4 **(261)**.

TRPV4 contributes to the development and maintenance of mechanical allodynia in the rodent neuropathic pain model of chronic compression of the DRG (CCD). TRPV4

expression is elevated at 7 to 28 days after CCD-type injury in rat, peaking at 7 days after such injury; and deletion of TRPV4 partially alleviates the mechanical allodynia resulting from such injury **(473)**. CCD in rat also results in thermal hyperalgesia to which TRPV4 may contribute and in which NF-κB also appears to be involved **(428, 430)**. Colchicine is a depolymerising agent of microtubules. Cholchicine, given intrathecally in rat, results in a dose-related and partial diminution in mechanical and thermal allodynia resulting from CCD, with the diminution in allodynia accompanied by a dose-related reduction in the level of TRPV4 expression. Colchicine also reduces TRPV4 currents in DRG neurons and in TRPV4-transfected HEK293 cells **(438)**. When administered intrathecally, colchicine reduces the effect of agonist-induced activation of TRPV4 on mechanical and thermal hyperalgesia in rats resulting from chronic compression injury to DRG neurons **(299)**.

Approximately half of the primary sensory neurons innervating the dura of the brain express TRPV4, with currents being generated by exposure to a hypotonic solution or some other TRPV4 activating stimulus. Activation of TRPV4 within the dura of rat results in face and hindpaw allodynia that is inhibited by the TRPV4 antagonist RN-1734 **(439)**.

(ii) Cancer

TRPV4 is expressed at a higher level in tumour-derived endothelial cells when compared with normal endothelial cells. TRPV4 is important in the migration of the former cell type but not in relation to the latter. Arachidonic acid results in actin being remodelled in tumour cells derived from human breast carcinoma. This, in turn, causes elevation of the level of TRPV4 found in the plasma membrane. This increased level of TRPV4 in the plasma membrane is believed to be important in promoting endothelial cell migration which results in angiogenesis and tumour growth **(122)**. On the other hand, the level of TRPV4 found in healthy or inflamed skin keratinocytes is reduced in premalignant lesions and in non-melanoma skin cancer, although whether this is a component of the mechanism of the disease remains unknown **(124, 231)**.

(iii) Brain injury and disease

TRP channels expressed in the brain may function in acquired brain disease, neurodegenerative disease and psychotic disorder **(294)**. TRPV4 is increased in astrocytes of

the CA1 region of the hippocampus in mouse at 7 days after hypoxia-induced ischaemia resulting from bilateral occlusion of the common carotid arterties for 15 minutes in hypoxic conditions. This is accompanied by astrogliosis. The 4α-PDD-induced response of astrocytes is also considerably increased in the aftermath of hypoxia-induced ischaemia **(48)**. Many by-products of stroke have the ability to activate TRPV4 and there is increasing evidence that excessive activation of TRPV4 as a result of stroke may mediate important aspects of the damage which results from stroke. There is evidence that stroke-induced neuronal injury is occasioned – at least in part – by upregulation of TRPV4 and its effect on NMDA receptor activation through its NR2B subunit and a related downregulation of the Akt signalling pathway **(186, 224**). In a mouse model of focal cerebral ischaemia, agonist-induced activation of TRPV4 increases NMDA current in hippocampal CA1 pyramidal neurons. This increased activation of NMDA receptors results from phosphorylation by CaMKII of the NR2B subunit. Intracerebroventricular injection of the TRPV4 antagonist, HC-067047, reduces brain infarction after 1 hour of occlusion of the middle cerebral artery **(224)**. Expression of TRPV4 is increased in the ipsilateral hippocampus from 4 to 48 hours after such occlusion in the mouse model and is at its maximum level at 18 hours after the event. Administration, at 24 hours after the event, of the TRPV4 antagonists, HC-067047 and ruthenium red, dose-dependently reduces brain infarction. When the TRPV4 agonist, GSK-1016790A , is administered by intracerebroventicular injection, hippocampal neuronal death is dose-dependently induced while there is an accompanying increase in the phosphorylation of the NR2B subunit of NMDA receptors. In addition, the p-Akt level declines while the p-ERK level rises after GSK-1016790A injection – effects which are inhibited by administration of an antagonist of NR2B **(186)**. Blockade of TRPV4 has also been found to reduce brain oedema in a mouse model of middle cerebral artery occlusion – an effect which has been proposed to result from inhibiting the activation of the matrix metalloproteinase 9 (MMP-9) and preventing tight junction protein loss **(187)**.

TRPV4 expressed in the endothelium of mouse cerebral arteries functions in mediating the dilation induced by acetylcholine in those arteries, but the functioning of these ion channels is impaired in mouse models of Alzheimer's disease. Experimental removal of the endothelium in posterior cerebral artery segments of wild type mice almost completely abolishes the dilation normally induced by a TRPV4 agonist or by acetylcholine. Dilation in response to a TRPV4 agonist or acetylcholine is impaired in arteries taken from mouse models of brain vascular pathology associated with Alzheimer's disease, namely, the mouse model of

enhanced amyloid β, that of cerebrovascular fibrosis, and that exhibiting both cerebrobascular fibrosis and enhanced amyloid β. Pre-treatment of wild type arteries with a TRPV4 antagonist prevents TRPV4 agonist-induced dilation and greatly reduces the dilation induced by acetylcholine, but has little effect on the dilation induced by acetylcholine on arterial segments obtained from the mouse models of Alzheimer's disease **(469)**. In the mouse model of enhanced amyloid β production, the soluble Aβ impairs blood vessels principally by enhancing oxidative stress. This causes a reduction in dilations mediated by NO, and impairment of signalling by TRPV4 in the endothelium and by KATP channels in smooth muscle. These functional impairments are found in the early stages of the disease and can be reversed by the use of antioxidants in animals expressing this model of Alzheimer's disease **(156)**.

11. TRPV4 channelopathies

Diseases caused by mutations of TRPV4 are described as TRPV4 channelopathies **(295)**. Mutations of TRPV4 result in both a range of skeletal dyplasias and peripheral neuropathies **(Figure 13)**. Mutant TRPV4 can result in skeletal dysplasia and degenerative neuropathy in the same patient. TRPV4 mutations expected to result in "neuropathies" can also cause skeletal dysplasias while TRPV4 mutations of the "skeletopathic" type can also result in neuropathies **(75)**. Evangelista and colleagues (2015) report a c.805C > T (p.Arg269Cys) TRPV4 mutation where the patient exhibits signs of skeletal dysplasia as well as scapuloperoneal spinal muscular atrophy. A c.184G > A (p.Asp62Asn) mutation of TRPV4 results in a clinical phenotype which is consistent with Charcot-Marie-Tooth disease (CMT) type 2C and in which basophic inclusions are found in the patient's muscle **(108)**.

(i) Skeletal dysplasias

Mutant TRPV4 ion channels result in a wide phenotypic spectrum of skeletal dysplasias **(206, 301)**. The extent to which the mutations to TRPV4 cause the channel's basal activity to be elevated is a predictor of the severity of the resulting skeletal dysplasia. Normal TRPV4 in Xenopus oocytes has a ~1% open probability (Po) without exposure to any channel activator. However, Po of most of the lethal metatropic dysplasia (MD) channels is close to 100%. Higher basal open probabilities are common to all mutant channels and this precludes additional channel gating in response to further exposure to channel activators. The

extent of TRPV4 channel over-activity correlates with the severity of the clinical disease **(237)**. An increased level of follistatin (FST) in chondrocytes caused by TRPV4 mutations has been identified as a probable contributing factor in skeletal dysplasias **(215)**. There is evidence that TRPV4 mutants which result in channelopathies with a "gain-of-function" phenotype lack sensitivity to PIP2 and lack the capacity to bind PIP2 to the TRPV4 ARD in the normal manner exhibited by TRPV4. Normally, the binding of phosphoinositide to an ARD of TRPV4 results in dampening channel activity **(395)**. When expressed in Xenopus oocytes, each of two TRPV4 mutants (L596P and W733R, respectively) activates normally in response to depolarization and exhibits outward rectification. On the other hand, these mutants possess increased basal open probabilities and exhibit reduced responses to GSK-1016790A, a TRPV4 agonist, which explains their "gain of function" phenotypes. In addition, current through the W733R mutant does not switch off during depolarization. The L596P mutation in TRPV4 is responsible for preventing bone development in human Kozlowski-type spondylometaphyseal dysplasia. The W733R mutation possesses a strong gain-of-function characteristic that reduces growth when this mutant is expressed in yeast (**399)**.

Five TRPV4-mediated skeletal dysplasias have been identified: (i) autosomal dominant brachyolmia type 3; (ii) spondylo-epymetaphyseal dysplasia Maroteaux pseudo Morquio type 2; (iii) spondylometaphyseal dysplasia Kozlowski type; (iv) parastremmatic dysplasa; and (v) metatropic dysplasia **(295)**.

Autosomal dominant brachyolmia type 3. Brachyolmia features a short trunk, scoliosis and mild short stature and has been attributed to point mutations in TRPV4 that encode R616Q and V620I substitutions, respectively **(331)**. It has been suggested that AD brachyolmia may not constitute a distinct entity but may rather be the mildest phenotypic expression of SMDK (spondylometaphyseal dysplasia Kozlowski-type) **(17)**.

Spondylo-epimetaphyseal dysplasia Maroteaux pseudo-Morquio type 2 (SEDM-PM2). This dysplasia is autosomal dominant and results in abnormalities of the vertebrae and long bones. During childhood, affected individuals develop short trunk and height and typically suffer from kyphoscoliosis, short fingers and toes, as well as "knock-knees" and osteoporosis **(295)**. R594 in exon 11 is a hotspot for SMDK mutations (**81)**.

Spondylometaphyseal dysplasia Kozlowski type (SMDK). This is an autosomal dysplasia which primarily results in abnormalities of the vertebrae and the metaphyses. During

childhood, affected individuals fail to grow to normal height and, indeed, remain very short in stature while also suffering from severe scoliosis. Frequently, the disease also afflicts the sufferer with early degenerative joint disease **(295)**.

Parastremmatic dysplasia. This is an autosomal dominal dysplasia which manifests itself in severe dwarfism, which is frequently accompanied by major twisting and distortion of the limbs. This dysplasia is manifest at birth and progresses rapidly during the early years. A failure in bone mineralization is believed to be responsible for the bony distortions effected by the disease **(295)**.

Metatropic dysplasia (MD). This is an autosomal dysplasia which can be lethal in some of its forms. When not lethal, MD is congenital, with affected individuals exhibiting short limbs relative to their trunk. During the course of childhood, subjects experience kyphoscoliosis with the result that their upper and lower limbs lengthen relative to their torso. "Metatropic" describes the alteration in skeletal phenotype which occurs. Lethal MD frequently manifests in foetal akinesia, severe contracutres of the joints and respiratory problems which result in perinatal death **(295)**. P799 in exon 15 is a hot codon for MD mutations, as four different amino acid substitutions have been observed at this codon **(81)**. In two cases of lethal MD, histology has identified obviously disrupted endochondral ossification with reduced numbers of hypertrophic chondrocytes and the presence of islands of cartilage within the zone of primary mineralization. This suggests that altered chondrocyte differentiation in the growth plate leads to the clinical features of this disease type which involves short extremities, a short trunk with progressive kyphoscoliosis and craniofacial abnormalities **(52)**.

(ii) Peripheral axonal neuropathies

TRPV4 mutants can also result in the development of degenerative diseases of the peripheral nerves. Three missense substitutions (R269H, R315W and R316C) in the TRPV4 gene affect the intracellular N-terminal ankyrin domain of TRPV4 and are responsible for: (i) congenital distal spinal muscular atrophies (SMA); (ii) scapuloperoneal SMA; and (iii) hereditary motor and sensory neuropathy 2C (HMSN 2C) **(295)**. These substitutions affect channel development, resulting in diminished expression of functional TRPV4 channels. TRPV4-induced Ca^{2+} ingress is diminished even when the receptor is subjected to recognised TRPV4 gating stimuli **(21)**.

Congenital spinal muscular atrophy (CDSMA). CDSMA is an autosomal dominant neuropathy involving a congenital loss of lower motor neurons which, fortunately, does not progress. However, it causes muscle weakness and atrophy as well as failure of certain neurological reflexes, such as the knee jerk reaction, in the lower body. Individual subjects will also frequently suffer from clubfoot and contractures on flexion of the knees and hips **(295)**.

Scapuloperoneal spinal muscular atrophy (SPSMA). SPSMA is an autosomal dominant neuropathy which involves a congenital decrease in the peroneus and shoulder blade muscles, accompanied by the phenomenon of "scapular winging." Individual subjects frequently also suffer from paresis of the vocal cords **(295)**. A c.805C > T (p.Arg269Cys) TRPV4 mutation has been identified where the patient exhibits signs of skeletal dysplasia as well as scapuloperoneal spinal muscular atrophy **(108)**.

Hereditary motor and sensory neuropathy 2C (HMSN 2C). HMSN 2C is also known as *Charcot-Marie-Tooth disease type 2 (CMT 2C).* CMT 2C is an autosomal dominant peripheral neuropathy which can affect the functioning of both motor and sensory neurons. Patients experience progressive weakness and atrophy of the muscles, especially of the distal muscles, together with loss of the sensation of touch. These signs and symptoms are often accompanied by paresis of the vocal cords, sensorineural hearing loss on both sides, urgency in micturition and event incontinence **(295)**. A c.184G > A (p.Asp62Asn) mutation of TRPV4 results in a clinical phenotype which is consistent with CMT 2C and in which basophilic inclusions are found in the patient's muscle **(108)**.

Landoure and colleagues (2010) expressed the view that the mutant TRPV4 mediate their pathological effect to occasion CMT 2C *via* "gain of function" instead of "loss of function" of the TRPV4 mutants. These authors identified two heterozygous missense mutations in the TRPV4 gene, C805T and G806A, resulting in the amino acid substitutions R269C and R269H, found in a specific area of the ankyrin repeats in TRPV4. In cells transfected with TRPV4, the CMT 2C mutations result in distinct cellular toxicity and elevated constitutive and activated channel currents, consistent with TRPV4 mutant "gain of function" being the author of the pathological condition **(213)**. Deng and colleagues (2010) identified mutant TRPV4 channels as the causes of scapuloperoneal SMA and CMT 2C and found increased channel activity to be a feature of these mutant TRPV4 channels **(91)**. Fecto and colleagues (2011) sought to resolve the apparent conflict between the findings and

conclusions of the Auer-Grumbach and the Deng/Landoure studies. The Fecto study **(113)** investigated the three mutant TRPV4 ion channels responsible for three independent axonal neuropathies. They found that these mutant channels had normal localisation but exhibited elevated Ca^{2+} channel activity which produces greater cytotoxicity. Moreover, their transfected HeLa cells demonstrated ~ 6-fold greater whole-cell currents than normal controls. These results suggest that the mechanism of action of the TRPV4 mutants involves a "gain of function" of the ion channel likely to lead to increased intracellular Ca^{2+} ingress. The mutant TRPV4 channels were found to exhibit a change in intrinsic gating that favours the open state, similar to that reported by Loukin and colleagues for the brachyolmia-causing R616Q mutation (**239)**. However, notwithstanding this increase in open probability in the mutants, single channel conductance remains unaffected. The Fecto study did, however, concede that the "gain of function" of the mutant channels could be the result of more effective channel insertion of mutant TRPV4 proteins **(113)**. The data obtained by Klein and colleagues (2011) in their study of CMT 2C also supports the proposition that "gain of function" of mutant TRPV4, rather than "loss of function" of these mutant channels, is pathologically important. They found two mutations in the ankyrin repeat domains of TRPV4 either of which can produce CMT 2C. Both mutant TRPV4 receptors have normal physiological localisation within the cells expressing them. Cells transfected with these mutant TRPV4 receptors have elevated intracellular Ca^{2+} levels and cell death can be reversed by ruthenium red **(202)**. Variations of mutations in TRPV4 occur in patients suffering from distal muscular atrophy (dSMA). Thus, in a girl with dSMA and vocal cord paralysis, a variant mutation (p.P97R) is found localised in the cytosolic N-terminus of TRPV4, upstream of the ankyrin-repeat domain, where the great majority of disease-associated mutations are found. This mutation results in a "loss of function" TRPV4 channel which is responsible for the disease. The mutation p.R232C can be found in patients with congenital dSMA and associated bone abnormalities **(121)**.

Another explanation which has been offered for the degeneration of peripheral motor and sensory nerves found in hereditary motor and sensory neuropathies and spinal muscular atrophy is that overexpression or over-activation of TRPV4 in developing peripheral nerves may occasion neurotrophic factor-induced neuritogenesis in those nerves resulting in these or other pathologies **(182)**.

(iii) Other TRPV4 channelopathies

Mutations encoding p.Gly270Val, p.Arg271Pro and p.Phe273Leu substitutions in the intracellular ankyrin-repeat domain of TRPV4 are responsible for familial digital arthropathy-brachydactyly (FDAB). This is primarily an inherited aggressive osteoarthropathy of the fingers and toes which results in the middle and distal phalanges becoming shortened. These mutant TRPV4 channels are not well expressed on the surface of the cell, exhibit a diminished Ca^{2+} ingress in response to TRPV4 agonists, and fail to respond to hypotonic stress **(211)**. Hence, the mutations found in these TRPV4 receptors, which result in the inherited osteoarthropathy, are of the "loss of function" variety.

When the human TRPV4 receptor is modified to include the TRPV4(P19S) polymorphism, it suffers a reduced response to hypotonic stress (compared with normal TRPV4) and to epoxyeicosatrienoic acid. This mutation has also been related to serum $[Na^+]$ and hyponatraemia in two non-Hispanic Caucasian male populations. $[Na^+]$ in the blood is reduced in persons with TRPV4(P19S) who also are 2.4 to 6.4 times more likely to suffer hyponatraemia when compared to normal subjects **(403)**.

12. Conclusions

TRPV4 is a Ca^{2+} permeable non-selective cation channel which is activated by a disparate array of stimuli, ranging from hypotonicity to heat and acid. TRPV4 has already been identified as subserving a great number of important physiological roles and various disease states are known to be attributable to the absence, or abnormal functioning, of this ion channel. The fact that this ion channel is constitutively expressed and capable of spontaneous activation in the absence of agonist stimulation is consistent with its involvement in mediating important homeostatic functions, including its already known importance in the regulation of $[Ca^{2+}]_i$ and control of intracellular and systemic water balance. Other striking features of TRPV4 are its widespread dissemination throughout the body and its capacity to interact with other proteins which, again, suggest that TRPV4 exercises a broad and diverse impact on physiological functioning. As such, TRPV4 appears to be an immensely important target for prophylactic and therapeutic pharmacological intervention. The antagonist RN-1734 completely inhibits both ligand-induced, and hypotonicity-induced, activation of TRPV4, without affecting the activity of other TRP channels, including TRPV1, TRPV3 and the melastatin type 8 TRP channel, making it a useful tool for laboratory use **(417).**

However, as with TRPV1, the difficulty in developing an antagonist of TRPV4 for therapeutic purposes resides in identifying a potent, selective and bioavailable small molecule which can target the TRPV4 channels of interest while saving harmless those TRPV4 channels whose functioning is necessary for non-pathological physiology. The likelihood is that systemic administration of an antagonist to TRPV4 will not offer a practical approach to targeting this receptor, and that any antagonist will have to be specifically targeted to affect only the delinquent receptors so as to avoid the occurrence of severe adverse effects. Unlike, for example, the serotonin receptor, of which multiple variants have been identified thereby enabling the development of more specific antagonists, there is no evidence of similar success being likely in the context of TRP channels. Since more selective blocking of identified delinquent variants of TRPV4 is not yet possible, another alternative may be to attempt to confine the antagonist to the locale of pathological interest by attaching it to another molecule which can be restricted to that locale. The practical utility of a drug which can inhibit TRPV4 is demonstrated by the use of butamben (n-butyl-p-aminobenzoic acid) as a local anaesthetic which provides analgesia. TRPV4 and TRPA1 are blocked by micromolar butamben (although butamben also activates TRPA1 at millimolar concentrations). Butamben attenuates acute animal pain behaviours in a TRPA1- or TRPV4-dependent manner **(26)** and its efficacy in accessing its target receptors through the skin can be considerably enhanced by using liposomal gel formulations, compared to plain gel formulations **(62)**. While a complete identification of the extent of the functional importance of TRPV4 remains to be elucidated, sufficient is known at this juncture to justify the application of resources to develop the capacity for effective pharmacological management of this ion channel for prophylactic and therapeutic purposes in humans.

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